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(54) Title: CALLASE-RELATED DNAs AND THEIR USE IN ARTIFICIAL MALE STERILITY

(57) Abstract

A tapetum-specific callase (β-1,3-glucanase) gene, designated A6, from *Brassica napus* and other members of the family *Brassicaceae* including *A. thaliana* has been discovered, isolated and cloned. The A6 gene encodes a 53 kDa callase enzyme of *Brassica napus* and equivalent proteins in other *Brassicaceae* family members. Coding sequence from the gene can be driven by an appropriate promoter to induce male sterility in plants. Further, the A6 promoter can be used to drive male sterility DNA such as that coding for a nuclease, protease or glucanase. Alternatively or in addition, male sterility can be achieved by disrupting the proper expression of the A6 gene, for example by transcribing RNA which is antisense to the RNA normally transcribed from the A6 gene, or by expressing DNA coding for a ribozyme specific for the A6 gene RNA transcript.

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CALLASE-RELATED DNAs AND THEIR USE IN ARTIFICIAL MALE STERILITY

This invention relates to recombinant, isolated and other synthetic DNA useful in male-sterility systems for plants. In particular, the invention relates to restorable male-sterility systems. Male-sterile plants are useful for the production of hybrid plants by sexual hybridisation.

Hybrid plants have the advantages of higher yield and better disease resistance than their parents, because of heterosis or hybrid vigour. Crop uniformity is another advantage of hybrid plants when the parents are extensively homozygous; this leads to improved crop management. Hybrid seed is therefore commercially important and sells at a premium price.

Producing a hybrid plant entails ensuring that the female parent does not self-fertilise. There have been many prior proposals, mechanical, chemical and genetic, for preventing self-pollination. Among the genetic methods is the use of anther-specific genes or their promoters to disrupt the normal production of pollen grains. An anther-specific promoter, for example, can be used to drive a "male-sterility DNA" at the appropriate time and in the right place. Male sterility DNAs include those coding for lytic enzymes, including those that lyse proteins, nucleic acids and carbohydrates. Glucanases are enzymes which break down carbohydrates.

In EP-A-0344029 (Plant Genetic Systems (PGS)) and WO-A-9211379 (Nickerson International Seed Company Limited) glucanase-coding DNA features among possible male-

sterility DNAs. Although many plant glucanases have been characterised and the genes cloned in some cases (eg defence-related "PR" glucanases), to date no glucanase with properties consistent with a role in microspore release has been reported. Microspore release is the process by which the immature microspores are liberated from a protective coat of $\beta(1,3)$ poly-glucan (callose) laid down by the microsporogenous cells before meiosis (Rowley, *Grana Palynol.*, **2**, 3-31 (1959); and Heslop-Harrison, *Can. J. Bot.* **46**, 1185-1191(1968) and *New Phytol.*, **67**, 779-786 (1968)). The anther-expressed glucanase responsible for the dissolution of this callose coat is known as callase. Callase is synthesised by the cells of the tapetum and secreted into the locule. The appearance of the enzyme activity is developmentally regulated to coincide precisely with a specific stage of microspore development.

The basis of the use of a glucanase as a sterility DNA lies in the fact that mis-timing of the appearance of callase activity is associated with certain types of male-sterility (Warmke and Overman, *J. Hered.* **63** 103-108 (1972)). Two types are recognised depending on whether the appearance of glucanase activity is premature or late. Since both types are found in nature, one important attraction of glucanase as a potential sterility DNA is that it already occurs in a natural system. Although plants that fail to produce active callase have not been described in nature, mutants of this type almost certainly occur. Failure to produce callase would prevent microspore-release, thereby causing pollen abortion and male-sterility. So, preventing callase expression would form the basis of a male-sterility system.

Several studies suggest that callase is probably different fr m other types of glucanases, such as the "PR" glucanases. For example, callase activity may be subject to both transcriptional and post- transcriptional control. This is suggested by the fact that there is a strong relationship between locule pH, callase activity, and the timing of microspore release. (Izhar and Frankel, *Theor. and Appl. Genet.* **41**, 104-108 (1971)). Locule pH and callase activity change coordinately in a developmentally regulated manner. In fertile *Petunia hybrida* anthers, the pH during meiosis is 6.8-7.0 and callase activity is undetectable. Following meiosis, at the tetrad stage, the locule pH drops in a precipitous fashion to 5.9-6.2 and callase activity increases sharply resulting in microspore release.

In certain male-sterile *Petunia* strains, the drop in pH and the appearance of callase activity are precocious and apparently result in the breakdown of microsporogenesis. Similarly, in another class of mutants, the drop in locule pH and the appearance of callase activity are both late and apparently result in the abortion of the microspores.

Thus, it appears that:

(1) the timing of the appearance of callase activity is critical for normal microspore development. (Presumably the abortion of prematurely released microspores indicates that they must reach a certain developmental stage before becoming capable of surviving without the protection of the callose coat);

(2) the decrease in locule pH parallels the appearance of callase activity; and

(3) the two events (production of callase activity and pH drop) are coordinately regulated in some manner.

The exact nature of the co-ordinate regulation of callase activity and pH is not known. The drop in pH may activate an otherwise fully functional enzyme (passive activation). Alternatively, the enzyme may be synthesised in an inactive form, rather like the zymogen of a protease, and activated as a consequence of some pH-dependent event such as the removal of an N-terminal or C-terminal addition (positive activation). The fact that callase, and possibly all glucanases, including PR-glucanases, has no detectable activity above pH 6.3, well below that encountered in the anther before microspore release may favour a passive activation theory.

However, since current assays for callase are crude and rely on the measurement of activity, it is impossible to say whether the enzyme is: i) produced before microspore release, but in a non-functional form for later activation; ii) synthesised in an active form but only at the precise time it is required; or iii) synthesised in advance in an active form, stored within the tapetal cells in some kind of vesicle, and released into the locule at microspore-release. The fact that pH drop and callase activity are so consistently correlated, even in cases where callase activity is found well before the normal time of microspore release, might indicate that the enzyme is synthesised in an inactive form in advance

of its requirement and that the pH drop is in some way r sponsible for its activation. The alternative is that the drop in pH triggers the synthesis of callase in the tapetal cells. The important point is that, without knowing which is correct, it is impossible to predict whether the expression of glucanases that are not callase will produce male sterility.

The fact that callase appears different in certain respects from previously characterised glucanases has three important consequences:

(1) glucanases, such as defence-related "PR" glucanases may not function efficiently under the conditions within the locule and may therefore not prove sufficiently useful as components of male sterility DNAs;

(2) in the event that such glucanases are active within the locule, maximum naturalness, in terms of mimicking existing types of male-sterile plants, would nevertheless demand the use of the authentic callase gene. In this respect a male sterility system based on the use of a callase gene would be superior to any previously described system; and

(3) systems based on preventing callase expression by destroying the callase mRNA using anti-callase mRNA, ribozymes or a callase anti-sense RNA require detailed knowledge of the nucleotide sequence of the callase mRNA.

The present invention is based on the discovery and identification of a callase gene in members of the family *Brassicaceae*. A cDNA derived from this gene in *Brassica napus* and a genomic version of the gene from *Arabidopsis thaliana* have been cloned. These and related DNAs (including the promoter of the callase gene) can be used in the construction of artificial male-sterility systems. Fertility can be restored in the F1 generation using antisense RNA, ribozymes and RNA-binding proteins.

According to a first aspect of the present invention, there is provided a recombinant or isolated DNA encoding an enzyme which has the activity of a callase enzyme particularly a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

In this specification, the gene encoding the 53 kDa callase enzyme of *B. napus* and equivalents of that gene in other members of the family *Brassicaceae* will be referred to as the A6 gene.

Preferred embodiments of this aspect of the invention include the gene encoding the 53 kDa callase enzyme from *B. napus* itself and the equivalent enzyme from *Arabidopsis thaliana* and their cDNAs.

The molecular weights quoted above are putative and derived from the number of amino acids believed to be present, as deduced from the DNA sequence. The 53 kDa protein encoded by the A6 gene of *B. napus* has 474 amino acids. It will therefore be appreciated that the molecular weights refer to the un-glycosylated protein. In addition, the effect on any other post-translational processing such as partial proteolysis is discounted.

Although the figure given above relate only to proteins of *B. napus*, those skilled in the art will readily be able to identify equivalent proteins from other members of the family *Brassicaceae*. For example, the equivalent A6 gene in *A. thaliana* encodes a putative protein of 479 amino acids in length having a calculated molecular weight of 53.7 kDa. Such equivalent genes may be identified by hybridisation studies, restriction fragment length polymorphism (RFLP) and other methods known in the art. Genes or other DNA sequences, whether natural, engineered or synthetic, encoding closely equivalent proteins may for example hybridise under stringent conditions (such as at approximately 35°C to 65°C in a salt solution of approximately 0.9 molar) to the *B. napus* A6 gene, or fragments of it of, for example, 10, 20, 50 or 100 nucleotides. A 15-20 nucleotide probe would be appropriate under many circumstances.

DNA sequences modified or differing from natural *Brassicaceae* A6 sequences are within the scope of the invention if, for example, they satisfy the above hybridisation criteria, or would do so but for the degeneracy of the genetic code.

The preferred A6 coding sequence described in this specification is from *Brassica napus* or *Arabidopsis thaliana* and can be isolated by methods known in the art, for example by (a) synthesising cDNA from mRNA isolated from the stamens of *B. napus* or *A. thaliana*, (b) isolating this cDNA, (c) using this cDNA as a probe to identify regions of the plant genome of a chosen member of the family *Brassicaceae* that encode stamen-specific mRNA and (d) identifying the upstream (5′) regulatory regions that contain the promoter of this DNA. This

procedure also demonstrates that probes based on, or derived from, the coding regions of a stamen-specific DNA from one species of plant may be used to isolate DNA sequences encoding stamen-specific mRNAs from other species.

Particularly preferred coding sequences are shown in Figure 1 (for the *B. napus* A6 gene) and Figure 4 (for the *A. thaliana* A6 gene) as will subsequently be described in the examples. Those skilled in the art will, with the information given in this specification, be able to identify with sufficient precision the coding regions and to isolate and/or recombine DNA containing them.

DNA in accordance with the first aspect of the invention is useful in the provision of male sterility systems. By operatively linking the DNA with a suitable promoter, it can be expressed at a time that would naturally be inappropriate, for example too early. Suitable promoters include tapetum-specific promoters other than the natural A6 promoter. Among the preferred promoters are those described and claimed in WO-A-9211379 and designated A3 and A9. In WO-A-9211379, the gene encoding the 12.9 kDa protein in *A. thaliana* and equivalents of that gene in other members of the family *Brassicaceae* are referred to as the A3 gene; the gene encoding the 11.6 kDa protein in *A. thaliana* and equivalents of that gene in other members of the family *Brassicaceae*, including the gene encoding a 10.3 kDa protein in *B. napus*, are referred to as the A9 gene. The contents of WO-A-9211379 are hereby incorporated by reference.

The discovery underlying the present invention can be harnessed in a number of other ways to provide a male-

st rility system. The A6 promoter can for example be used to drive male-sterility DNA, which does not need to be specific.

According to a second aspect of the invention, there is provided a recombinant or isolated DNA sequence comprising a promoter which naturally drives the expression of a callase enzyme, particularly a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

Because of the natural specificity of the regulation of expression of the A6 gene, it is not necessary for the A6 promoter to be linked to specific disrupter DNA to provide a useful male-sterility system (although it can be); non-specific disrupter DNA can be used.

A6 promoters from other members of the family *Brassicaceae* and modified A6 promoters can be used, and if necessary located or identified and isolated as described above for the A6 coding sequences, *mutatis mutandis*. Again, preferred promoters are from *B. napus* and *A. thaliana* and used naturally to drive the coding sequences shown in Figures 1 and 4, which will be described later.

A6 promoter-containing DNA in accordance with the invention can, as indicated above, be used to confer male sterility on plants, particularly those belonging to the family *Brassicaceae*, in a variety of ways as will be discussed below. In an important embodiment of the invention, therefore, a promoter as described above is operatively linked to DNA which, when expressed, causes male sterility.

Since an effective sterility system is complete, propagation of the seed parent must proceed either by asexual means or via the pollination of the male-sterile by an isogenic male-fertile line, and the subsequent identification or selection of male sterile plants among the offspring. Where vegetative propagation is practical, the present invention forms a complete system for hybrid production. Where fertility restoration is necessary to produce a seed crop, the present invention forms the basis of a new male sterility system. In some seed crops where the level of cross pollination is high, seed mixtures may enable restoration to be bypassed. The male sterility will be particularly useful in crops where restoration of fertility is not required, such as in the vegetable *Brassica* spp., and such other edible plants as lettuce, spinach, and onions.

DNA in accordance with the invention and incorporating the A6 promoter can drive male sterility DNA thereby producing male sterile plants, which can be used in hybrid production. The promoters are highly tapetum-specific and so the sterility DNA is only expressed in the tapetum. The control of expression is very strong and the DNA is not expressed in other cells of the plant. The system prevents the production of viable pollen grains. All transformed plants and their progeny are male sterile; there is no problem with meiotic segregation.

A construct comprising a promoter operatively linked to a male sterility DNA can be transformed into plants (particularly those of the genus *Brassica*, but also other genera such as *Nicotiana* and *Hordeum*) by methods which may be well known in themselves. This transformation results in the production of plants, the cells of which

contain a foreign chimeric DNA sequence composed of the promoter and a male sterility DNA. Male-sterility DNA encodes an RNA, protein or polypeptide which, when produced or over-produced in a stamen cell of the plant, prevents the normal development of the stamen cell.
The A6 promoter may be used to drive a variety of male sterility DNA sequences which code for RNAs, proteins or polypeptides which bring about the failure of mechanisms to produce viable male gametes. The invention is not limited by the sequence driven, but a number of classes and particular examples of male sterility promoter-drivable sequences are preferred.

For example, the drivable male sterility DNA may encode a lytic enzyme. The lytic enzyme may cause degradation of one or more biologically important molecules, such as macromolecules including nucleic acid, protein (or glycoprotein), carbohydrate and (in some circumstances) lipid.

Ribonuclease (such as RNase T1 and barnase) are examples of enzymes which cause lysis of RNA. Examples of enzymes which lyse DNA include exonucleases and endonucleases, whether site-specific such as EcoRI or non-site-specific.

Glucanases other than the callase to whose coding sequence a promoter of the invention is naturally linked represent examples of enzymes which cause lysis of a carbohydrate. The enzyme glucanase (callase) is naturally produced in anthers where it functions to release the young microspores from a protective coat of poly-glucan laid down before meiosis. The appearance of the enzyme activity is developmentally regulated to coincide with the correct stage of microspore

development. One important attraction of glucanase as a potential sterility DNA is that plants are found in nature that are male-sterile due to mutations causing mistiming of callase expression and the destruction of the microspores. Two types are recognised depending on whether the appearance of callase activity is premature or late. The expression of many genes, including those expressed within the anther, exhibit various patterns of temporal regulation. Therefore, in order to use callase as a sterility DNA, the promoter chosen to drive expression of the gene must provide an appropriate developmental regulation of glucanase activity, preferably by mimicking the pattern of expression found in association with natural male-sterility. One means of achieving male sterility is to isolate the promoter from a tapetum-specific gene with the same pattern of expression as found for glucanase activity in male-sterile mutant plants. Since late expression of a glucanase is unlikely to produce sterility in plants with a functional anther glucanase gene, the sterility factor would require a promoter capable of driving transcription before the appearance of normal glucanase activity. In the RM cms mutant of *Petunia* (Izhar, S. and Frankel, R. *Theor. Appl. Genet.*, **41** 104-108 (1971)) callase expression within the anther first appears at the end of meiotic prophase, and increases to a maximum by the completion of meiosis. This pattern of expression contrasts with that in normal *Petunia* plants, where glucanase activity within the anthers appears concomitantly with the breakdown of the tetrads and the release of the young microspores. The aberrant pattern of callase activity found in the cms mutant is thought to be responsible for the destruction of the microspores and male sterility. Thus, to mimic this mutation using a

sterility DNA encoding a glucanase enzyme requires a promoter capable of driving transcription of the male sterility DNA within the anthers, and preferably within the tapetum, during the phase of anther development between prophase of meiosis and the appearance of the tetrad of microspores; the A3 and A9 promoters discussed above are therefore well suited to drive this gene. A tapetum-specific (or at least anther-specific) promoter is also advantageous since $\beta(1,3)$-glucans are found elsewhere within plants, for example in phloem sieve elements, where they presumably perform essential functions.

The spatial regulation of the enzyme should also ensure access to the target cells. Secretion into the locular space is ensured by the provision in a preferred embodiment, of the natural or any other suitable signal sequence in a translational fusion with the glucanase coding sequence.

DNA encoding glucanase is advantageous as male sterility DNA, as it has no product which is cytotoxic outside the target cell. Glucanase as a male sterility DNA mimics natural systems and is inherently less destructive than for example ribonuclease, and so does not present such a problem if 'leakage' occurs into other cells.

Actinidin is an example of a protease, DNA coding for which can be suitable male sterility DNA. Other examples include papain zymogen and papain active protein.

Lipases whose corresponding nucleic acids may be useful as male sterility DNAs include phospholipase A_2.

Male sterility DNA does not have to encode a lytic enzyme. Other examples of male sterility DNA encode enzymes which catalyse the synthesis of phytohormones, such as isopentyl transferase, which is involved in cytokinin synthesis, and one or more of the enzymes involved in the synthesis of auxin. DNA coding for a lipoxygenase or other enzymes having a deleterious effect may also be used.

Other male sterility DNAs include antisense sequences. Introducing the coding region of a gene in the reverse orientation to that found in nature can result in the down-regulation of the gene and hence the production of less or none of the gene product. The RNA transcribed from antisense DNA is capable of binding to, and destroying the function of, a sense RNA version of the sequence normally found in the cell thereby disrupting function. Examples of such anti-sense DNAs are the anti-sense DNAs of the A6 gene produced in the anther under control of the A6 promoter. Since this gene is normally expressed in the tapetum, antisense to it may be expected to disrupt tapetal function and result in male sterility.

It is not crucial for antisense DNA solely to be transcribed at the time when the natural sense transcription product is being produced. Antisense RNA will in general only bind when its sense complementary strand, and so will only have its toxic effect when the sense RNA is transcribed. Antisense DNA corresponding to some or all of the DNA encoding the A6 gene product may therefore be produced not only while the A6 gene is being expressed. Such antisense DNA may be expressed constitutively, under the control of any appropriate promoter.

According to a further aspect of the invention, therefore, there is provided antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of the strand of DNA that is naturally transcribed in a gene encoding a callase enzyme, such as a 53 kDa callase enzyme in *B. napus* or an equivalent protein in another member of the family *Brassicaceae*.

Antisense DNA in accordance with this aspect of the invention may be under the control of any suitable promoter which permits transcription during, but not necessarily only during, tapetum development. As indicated above, the promoter may therefore be constitutive, but the use of a tapetum-specific promoter such as A3 or A9 as described above in relation to the second aspect of the invention is certainly not excluded and may be preferred for even greater control. Such antisense DNA would generally be useful in conferring male sterility on members of the family *Brassicaceae*.

A still further example of male sterility DNA encodes an RNA enzyme (known as a ribozyme) capable of highly specific cleavage against a given target sequence (Haseloff and Gerlach *Nature* **334** 585-591 (1988). Like antisense DNA, ribozyme DNA (coding in this instance for a ribozyme which is targeted against the RNA encoded by the A6 gene) does not have to be expressed only at the time of expression of the A6 gene. Again, it may be possible to use any appropriate promoter to drive ribozyme-encoding DNA, including one which is adapted for constitutive expression.

According to a further aspect of the invention, there is therefore provided DNA encoding a ribozyme capable of

specific cleavage of RNA encoded by a gene encoding a callase enzyme, such as a 53 kDa callase enzyme in *B. napus* or an equivalent protein in another member of the family *Brassicaceae*. Such ribozyme-encoding DNA would generally be useful in conferring male sterility on members of the family *Brassicaceae*.

In preferred embodiments of DNA sequences of this invention, including those comprising the A6 promoter-male sterility DNA construct, 3′ transcription regulation signals, including a polyadenylation signal, may be provided. Preferred 3′ transcription regulation signals are derived from the Cauliflower Mosaic Virus 35S gene. It should be recognised that other 3′ transcription regulation signals could also be used.

The antisense nucleic acid and ribozyme-encoding nucleic acid described above are examples of a more general principle: according to another aspect of the invention, there is provided DNA which causes (for example on its expression) selective disruption of the proper expression of the callase, or in preferred embodiments A6, gene.

Recombinant DNA in accordance with the invention may be in the form of a vector. The vector may for example be a plasmid, cosmid or phage. Vectors will frequently include one or more selectable markers to enable selection of cells transfected (or transformed: the terms are used interchangeably in this specification) with them and, preferably, to enable selection of cells harbouring vectors incorporating heterologous DNA. Appropriate start and stop signals will generally be present. Additionally, if the vector is intended for expression, sufficient regulatory sequences to drive expression will

be present; however, DNA in accordance with the invention will generally be expressed in plant cells, and so microbial host expression would not be among the primary objectives of the invention, although it is not ruled out. Vectors not including regulatory sequences are useful as cloning vectors.

Cloning vectors can be introduced into *E. coli* or another suitable host which facilitate their manipulation. According to another aspect of the invention, there is therefore provided a host cell transfected or transformed with DNA as described above.

DNA in accordance with the invention can be prepared by any convenient method involving coupling together successive nucleotides, and/or ligating oligo- and/or poly-nucleotides, including *in vitro* processes, but recombinant DNA technology forms the method of choice.

Ultimately, DNA in accordance with the invention (whether (i) A6 promoter plus male sterility gene, (ii) antisense DNA to A6 gene or ribozyme DNA targeted to A6 RNA) will be introduced into plant cells, by any suitable means. According to a further aspect of the invention, there is provided a plant cell including DNA in accordance with the invention as described above.

Preferably, DNA is transformed into plant cells using a disarmed Ti-plasmid vector and carried by *Agrobacterium* by procedures known in the art, for example as described in EP-A-0116718 and EP-A-0270822. Alternatively, the foreign DNA could be introduced directly into plant cells using an electrical discharge apparatus. This method is preferred where *Agrobacterium* is ineffective, for example

where the recipient plant is monocotyledenous. Any other method that provides for the stable incorporation of the DNA within the nuclear DNA of any plant cell of any species would also be suitable. This includes species of plant which are not currently capable of genetic transformation.

Preferably DNA in accordance with the invention also contains a second chimeric gene (a "marker" gene) that enables a transformed plant containing the foreign DNA to be easily distinguished from other plants that do not contain the foreign DNA. Examples of such a marker gene include antibiotic resistance (Herrera-Estrella *et al*, *EMBO J.* **2**, 987-995 (1983)), herbicide resistance (EP-A-0242246) and glucuronidase (GUS) expression (EP-A-0344029). Expression of the marker gene is preferably controlled by a second promoter which allows expression in cells other than the tapetum, thus allowing selection of cells or tissue containing the marker at any stage of regeneration of the plant. The preferred second promoter is derived from the gene which encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. However any other suitable second promoter could be used.

A whole plant can be regenerated from a single transformed plant cell, and the invention therefore provides transgenic plants (or parts of them, such as propagating material) including DNA in accordance with the invention as described above. The regeneration can proceed by known methods. When the transformed plant flowers it can be seen to be male sterile by the inability to produce viable pollen. Where pollen is produced it can be confirmed to be non-viable by the inability to effect seed set on a recipient plant.

Male fertility curtailed by means of the present invention may be r stored by an appropriate restoration system, whose nature will correspond to the particular manner used to render the plant male-sterile. Specific and preferred restoration systems described below are based on different mechanisms: antisense RNA and ribozymes.

antisense RNA: where the disrupter gene encodes a non-anther mRNA, such as the mRNA for the protein actinidin, restoration is provided by crossing into the male-sterile plant a gene encoding an anti-sense RNA specific to the disrupter mRNA driven by a tapetum-specific promoter with the appropriate temporal regulation. This will lead to the destruction of the sense mRNA and restore fertility. This approach is not applicable where the disrupter is prematurely expressed callase since expression of a callase anti-sense RNA will lead to the destruction of both the target disrupter callase mRNA and the normal callase mRNA which is required for microspore release and the production of viable pollen grains. Thus fertility would not be restored.

ribozymes: this approach is more generally applicable since the target site for ribozymes is small and therefore can be engineered into any mRNA. This allows in principal any introduced mRNA to be specifically targeted for destruction. Thus mRNAs encoding non-specific disrupter functions such as actinidin are destroyed and fertility restored by crossing in a gene encoding ribozymes specific to the actinidin mRNA. Where the disrupter is callase, restoration is achieved by crossing in genes encoding ribozymes specific to a short synthetic sequence introduced into the non-translated

leader of the prematurely expressed disrupter callase mRNA. Since this sequence is not present in the normal unmodified callase mRNA correctly timed callase activity is unaffected and fertility is restored.

Some preferred features of the invention have been described only in relation to one aspect of it. It will be appreciated that preferences extend to all aspects of the invention *mutatis mutandis*.

The invention will now be illustrated by a number of non-limiting examples, which refer to the accompanying drawings, in which:

Figure 1 shows the DNA sequence of the *B. napus* cDNA A6 together with the deduced protein sequence of the ORF contained in A6;

Figure 2 shows an alignment of the deduced primary structure of the *B. napus* and *A. thaliana* A6 genes with the primary structure of previously described glucanases; the following is a key:

Bn A6: 53 kDa anther-specific protein of *B. napus*

At G62: Corresponding A6 protein from *A. thaliana*

NPGLUC: Tobacco β-1,3 glucanase (basic) (De Loose *et al.*, *Gene* **70** 13-23, (1988)

BEAN: Bean β-1,3 glucanase Edington *et al.*, *Plant Mol. Biol.* **16**, 81-94 (1991)

PR-Q: Tobacco β-1,3 glucanase (extra- cellular) (Payne *et al.*, *Plant Mol. Biol.* **15** 797-808 (1990)

BARLEY: Hoj *et al.*, *Plant Mol. Biol.* **13**, 31-42 (1989);

Figure 3 shows a restriction enzyme map of the *A. thaliana* genomic clone G6.2. Only relevant sites are shown and these may not be unique in G6.2. The position of the coding region of A6 is indicated as a filled box. Also the extent of the insert cloned into the plasmid pDIH9 is shown;

Figure 4 shows the DNA sequence and putative primary structure of the *A. thaliana* A6 gene. The underlined sequence conforms to a TATA box motif;

Figure 5a shows a comparison of the DNA sequences of the *B. napus* cDNA A6 with the *A. thaliana* A6 gene. The underlined trinucleotides indicate the start and stop positions of the A6 coding sequences;

Figure 5b shows a comparison of the putative polypeptide encoded by *B. napus* cDNAs A6 with that encoded by the *A. thaliana* A6 gene;

Figure 6 shows the construction of a chimeric gene containing a transcriptional fusion between the A6 promoter and an *E. coli* gene encoding β-glucuronidase;

Figure 7a refers to Example 3a and shows the construction of a chimeric gene containing a transcriptional fusion between the A6 promoter and a sequence encoding mature barnase;

Figure 7b refers to Example 3b and shows the construction of a chimeric gene containing a transcriptional fusion between the A6 promoter and a sequence encoding actinidin;

Figure 8 shows the construction of chimeric genes between tapetum-specific promoters and *A. thaliana* callase: a) Transcriptional fusion between the A9 promoter and callase; b) Transcriptional fusions between the A9 and A6 promoters with callase lacking the sequence encoding the protein's C-terminal extension; and

Figure 9 shows the construction of plasmids pWP80, pWP83 and pWP88.

Abbreviations used for restriction enzymes in the drawings are:

B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; Hd, *Hind*III; K, *Kpn*I; N, *Not*I; Nc, *Nco*I; Np, *Nsp*I; Nr, *Nru*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; S, *Sst*I; Sa, *Sal*I; Sp, *Sph*I; Sm, *Sma*I; SII, *Sac*II; X, *Xho*I; Xb, *Xba*I.

Example 1 - Isolation of a cDNA encoding the anther-specific β(1,3) glucanase (callase) from *Brassica napus* and isolation of the corresponding gene from *Arabidopsis thaliana*

Anther-specific cDNAs have been isolated by differential screening of *Brassica napus* cDNA libraries constructed from RNA extracted from dissected anthers as described below (Scott *et al*, *Plant Mol. Biol.* in press). cDNA clone A6 was isolated from a library constructed from anthers that were 1.8-2.0 mm in length. This library was constructed in the vector Lambda ZapII (Stratagene). The A6 cDNA was used as a probe to isolate homologous genes from an *A. thaliana* genomic library constructed in the vector Lambda Dash (Stratagene).

Materials and methods

Plant material. All seeding material for nucleic acid isolation was obtained from 2-3 week old plants grown in a controlled environment growth cabinet with 18h photoperiod at 24°C. Seedling RNA for differential screening and Northern blot analysis was obtained from *B. napus oleifera* var. "Topaz". Male fertile buds were collected from field grown plants of *B. napus oleifera* var. "Lictor" (Nickersons Seeds, Cambridge, UK). Male-sterile buds were obtained from field grown *B. napus* var. CMS "ogura" (Nickersons Seeds, Cambridge, UK) plants.

Dissection of anthers. For cDNA library construction, flower spikes were quickly harvested and kept at 4°C until required, but no longer than 5h. Anthers were dissected from appropriately sized buds using fine forceps and immediately frozen in liquid nitrogen.

Collection of buds. Large samples of complete whorls of buds, at a stage immediately prior to the opening of first flowers, were frozen in liquid nitrogen and stored at -80°C.

Cytological staging of anthers and buds. The developmental stage of buds of predetermined length was assessed by light microscopic examination of sporogenous cells, microspores or pollen grains extruded from whole anthers squashed in the presence of aceto-orcein or acridine orange. Accurate determination of bud length was performed using a low-powered light microscope equipped with a calibrated eyepiece graticule. Bud lengths stated were measured from the base of the pedicle to the tip of the outermost sepal.

RNA isolation and analysis. Material intended for low resolution Northern dot blot analysis or for mRNA isolation was ground to a fine powder in a mortar cooled with liquid nitrogen. Total RNA was isolated from the powder using a phenol based method as described previously (Draper *et al* "Plant Genetic Transformation and Gene Expression: A Laboratory Manual", Blackwell Scientific Publishers, Oxford (1988)). Poly(A)$^+$ RNA was purified by two rounds of oligo(dT)-cellulose chromatography essentially as described in the Maniatis *et al* manual. RNA for high resolution dot blots was isolated according to the method of Verwoerd et al, *Nuc. Acids Res.* 17 2362 (1989)).

cDNA library construction and screening. cDNAs were synthesised from poly(A)$^+$ RNA using (Amersham) or (Pharmacia) cDNA synthesis kits, according to the manufacturers instructions. cDNAs were ligated into *Eco*RI cleaved dephosphorylated lambda Zap I (Stratagene) ("sporogenesis" library) or lambda Zap II (Stratagene) ("microspore-development" library) and packaged using Amersham *in vitro* packaging extracts. (When cloning into lambda Zap II, *Eco*RI linkers (Pharmacia Ltd) were used; these linkers also contain internal *Not*I sites, so the entire cDNA can be recovered as a *Not*I fragment, providing that the cDNA contains no internal *Not*I sites.) Clones were screened differentially, on duplicate HYBOND-N filters (Amersham) with [^{32}P]-labelled single-stranded cDNA probe prepared from either the appropriate anther poly(a)$^+$ RNA or seedling poly(A)$^+$ RNA according to Sargent *Methods in Enzymol.* 152 423-432 (1987)). (The expression HYBOND-N is a trade mark.)

RNA dot and gel blots. Total RNA f r dot-blots was spotted onto HYBOND N (Amersham) according to the manufacturers instructions. Northern gels were run and RNA transferred to HYBOND-N according to Fourney (*BRL Focus* **10** 5-7 (1988)). Hybridisation and washing of HYBOND-N filters was according to manufacturers instructions.

In situ hybridisation. For embedding and sectioning *B. napus* buds were frozen in CRYO-M-BED (TAAB Laboratories Equipment Ltd). (The expression CRYO-M-BED is a trade mark.) Sections were cut nominally 10 μm thick, mounted on subbed slides (Van Prooijen-Knegt et al *Histochemical J.* **14** 333-344 (1983)) fixed in 4% paraformaldehyde and dehydrated. [^{35}S]rUTP (>1000 Ci/mmol, Amersham SJ.1303) labelled sense and anti-sense RNA probes were transcribed from the T3 and T7 promoters of BLUESCRIPT SK$^-$ (Stratagene), in which the cDNAs are cloned. (The expression BLUESCRIPT SK$^-$ is a trade mark.) Following transcription, probes were cleaved by alkaline hydrolysis to generate probe fragments approximately 150bp in length. The hybridisation solution was 50% formamide, 300mM NaCl, 10mM Na_2HPO_4 pH 6.8, 10mM Tris-HCl pH 7.5, 5mM EDTA, 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 10mM dithiothreitol, 10% dextran sulphate, 0.7mg/ml *E. coli* tRNA, 50-100ng/ml probe stock ($6.7x10^5$ cpm/ng probe). Sections were hybridised in 30 μl hybridisation solution at 50°C for 16h. Slides were washed 3x1h at 50°C in 50% formamide, 300mM NaCl, 10mM Na_2HPO_4 pH 6.8, 10mM Tris-HCl pH 7.5 and then rinsed in RNase A buffer to remove formamide. RNase A treatment, (150 μg/ml RNase A in 500mM NaCl, 10mM Tris HCl pH 7.5), was carried out at 37°C for 1h. The slides were then washed twice in 2xSSC (0.3M NaCl, 0.03M Na citrate, pH

7.0) at 65°C for 30 min, dehydrated through graded alcohols and dried. For autoradiography, slides were dipped at 45°C in ILFORD K5 nuclear track emulsion (1g/ml in 1:59 glycerol:water mix). (The expression ILFORD K5 is a trade mark.) Exposure time was between 2 and 14 days. Development was in KODAK D19. (The expression KODAK D19 is a trade mark.) Following development sections were stained with methylene blue and made permanent.

a) Analysis of the *B. napus* A6 cDNA.

Northern hybridisation analysis using RNA extracted from *B. napus* anthers, pollen, carpels and seedlings indicated that A6 was only expressed in anthers of length 1.5-2.0mm with maximal expression at about 1.8mm. Thus A6 temporal expression spans the period in anther development when the microsporocytes are in meiotic division to early microspore interphase. The A6 cDNA is 1532 bp in length and contains an open-reading frame (ORF) extending from position 1- 1424 bp (Figure 1) suggesting that this clone is not full-length. The estimated size of *B. napus* A6 mRNA from Northern gel blots is about 1700 bp, again suggesting that this clone is not full-length. The ORF encodes a polypeptide of 474 amino-acids with a molecular weight of 53 kda, which is homologous to pathogenesis-related (PR) and other previously characterised $\beta(1,3)$-glucanases (Figure 2) strongly suggesting that A6 encodes the anther-specific $\beta(1,3)$ glucanase (callase). As will be described in Example 6 below, the production of anti-sense RNA to the A6 transcript in anthers of transgenic plants produces male sterile plants. These plants have a phenotype that is consistent, at the biochemical and cytological level, with the assertion that A6 encodes callase.

The alignment of A6 with β(1,3) glucanases shows that A6 is significantly larger due to the presence of a long C-terminal extension, the beginning of this extension corresponding to the C-terminus of mature β(1,3) glucanase enzymes. The level of homology of A6 to other glucanases although very significant (33 % identity over the region of homology) is however lower than that seen between the most divergent previously isolated β(1,3) glucanases (51% identity). Thus the A6 protein is not recognised by antibodies raised to the acidic PR glucanase of tomato or to the basic hormonally induced β(1,3) glucanase of tobacco. No hybridisation is observed to *B. napus* anther RNA or to the *B. napus* cDNA library using ^{32}P labelled *A. thaliana* genomic glucanase sequences (provided by F. Ausubel) or using ^{32}P labelled pGL43, a clone containing a basic β(1,3) glucanase from *N. tabacum* (Shinshi *et al. Proc. Natl. Acad. Sci. USA* **85**, 5541-5545 (1988)). Thus it is not possible to clone anther-specific callases by using available β(1,3) glucanase sequences or antibodies. However the alignment of A6 with other glucanases shown in Figure 2 enables the identification of amino-acids that are likely to be conserved in all glucanases. This allows the design of oligonucleotides that will be specific probes for β(1,3) glucanases and thus enable the cloning of the anther-specific glucanase cDNAs or genes from other plant species. Callase can be distinguished from other β(1,3) glucanases by virtue of its unique spatial and temporal pattern of expression coupled with the possession of a longer C-terminal extension than other β(1,3) glucanases.

b) Isolation and characterisation of homologous genes to A6 in *A. thaliana*.

Two genomic clones were isolated from an *A. thaliana* genomic library that hybridised to the *B. napus* A6 cDNA. One, G6.2, was analysed in detail (Figure 3). A 3.2 kb *Eco*RI fragment was subcloned into *Eco*RI-cut pTZ18U (Pharmacia) forming pDIH9 (Figure 3), and the coding region of A6 and 881 bp upstream was sequenced (Figure 4). Comparison of the *B. napus* and *A. thaliana* A6 sequences showed that they were 85% identical in the coding regions (Figure 5a) at the nucleotide level and 83% identical at the protein level (Figure 5b). The sequence alignment shows that the ORF encoded by the *B. napus* cDNA is almost full-length and probably lacks about 5 residues at the N-terminus. The *A. thaliana* A6 gene encodes a product of 479 amino-acids with a predicted molecular weight of 53.7 kDa. The A6 proteins have a hydrophobic N-terminal sequence that conforms to the rules defined by von Heijne, (*J. Mol. Biol.* **184**, 99-105 (1985)) for signal sequences. Callase is secreted from the tapetum into the anther locule and therefore should possess such a sequence.

The other genomic clone isolated (G6.1) was partially sequenced and was shown to be virtually identical to G6.2 both within the A6 coding region and also within the putative A6 promoter region.

Example 2 - The use of the A6 promoter to drive the expression of Glucuronidase in anthers of *Arabidopsis thaliana*, *Brassica napus*, *Hordeum vulgare*, *Nicotiana tabacum* and *Zea mays*

To demonstrate that the putative promoter region of A6 is capable of driving the expression of a foreign gene in *A. thaliana*, *B. napus*, *H. vulgare* and *N. tabacum* a

transcriptional fusion of the promoter was made to th *Escherichia coli* gene encoding β-glucuronidase (GUS). An 844 bp *Eco*RI-*Nsp*I fragment (position 1-884 bp in Figure 4) containing the putative A6 promoter is excised from pDIH9 and the ends rendered blunt with Klenow. This fragment is cloned into the *Sma*I site of pBluescript forming pDIH10 (Figure 6). The A6 promoter is then cloned as a *Sal*I, *Bam*HI fragment into pBI101.1 (Jefferson *et al.*, *EMBO. J.* **6**, 3901-3907 (1987)) forming pDIH11 (Figure 6). This plasmid contains the A6 promoter transcriptionally fused to GUS. pDIH11 is then transformed into *N. tabacum*, *A. thaliana*, *B. napus*, *H. vulgare* and *Z. mays* using standard transformation techniques. Transformation of *H. vulgare* is achieved using a microprojectile gun. Analysis of transformed plants demonstrates that GUS activity is localised to anther tissues, specifically to tapetal cells. The temporal regulation of GUS activity is identical to the temporal expression observed for the A6 genes as described in Example 1. The A6 promoter drives transcription in tapetal cells through a period commencing at the meiocyte stage of development and terminating during early microspore interphase.

The use of the A6 promoter to create male sterile plants.

Tapetum-specific promoters can be employed in a variety of ways to generate male sterile plants. For example, male sterility can be achieved by using the tapetum-specific promoter to express antisense and sense transcripts corresponding to tapetal messages (see Example 6), drive the premature expression of glucanase activity (see Example 4) and drive the expression of cytotoxic agents such as proteases and nucleases.

Example 3 - Construction of a chimeric A6-Barnase gene and a chimeric A6-actinidin gene and their expression in transgenic plants

To demonstrate the utility of the A6 promoter it is used to drive the expression of the RNAase, barnase, and the protease, actinidin, in tapetal cells.

Example 3A - Construction and expression in transgenic plants of chimeric gene fusion between the tapetum-specific A6 promoter and barnase

To demonstrate the utility of the A6 promoter it is used to drive the expression of the RNAase, barnase, in tapetal cells. Use of the barnase gene to create male sterile plants has been described in patent application EP-A-0344029 (Plant Genetic Systems) and has been published by Mariani *et al*. *Nature* **347**, 737-741.

The oligonucleotide primers
5' GGGTCTAGACCATGGGCACAGGTTATCAACACGTTTGACGGG 3' and
5' GTAAAACGACGGCCAGTGCC 3'
are used in a polymerase chain reaction (PCR) to generate a fragment encoding barstar and the mature barnase product from the plasmid pTG2 (Horovitz *et al*. *J. Mol. Biol*. **216**, 1031-1044 (1990)). The first primer is homologous to nucleotides 195-221 bp of Figure 1 in Hartley R.W. *J. Mol. Biol*. **202**, 913-915. The second primer is homologous to a sequence immediately next to the *Hin*dIII site of pTZ18U (Pharmacia). The PCR fragment is digested with *Xba*I and cloned into *Xba*I-cut pDIH12 forming pDIH13 in which the A6 promoter is transcriptionally fused to the mature barnase sequence (Figure 7). (pDIH12 is constructed by cloning the *Kpn*I, *Xba*I fragment of pDIH10 (Figure 6) into *Kpn*I, *Xba*I-cut

pWP80 (see below and WO-A-9211379).) This gene fusion is transferred to pBin19 (Bevan *et al* 1984) by ligating the EcoRV fragment of pDIH13 to *SmaI*-cut pBin19. The pBin19 derivative plasmid is transformed into *N. tabacum*, *B. napus*, *H. vulgare* and *Z. mays* where expression of barnase in transgenic plants results in the degradation of the tapetal and microsporocyte cells of the anther causing male sterility.

Plasmids pWP80, pWP83 and pWP88

pWP80, an intermediate vector designed to express sense and anti-sense RNA using the *A. thaliana* tapetum-specific A9 promoter, was constructed as follows. The isolation of the *A. thaliana* tapetum-specific A9 promoter is described in WO-A-9211379. To construct pWP80, pWP72 (WO-A-9211379) is digested with *Xba*I and religated, thus removing the *Bam*HI site in the polylinker and forming pWP78 (Figure 9). The *Kpn*I, *Sst*I (the *Sst*I end rendered blunt with Klenow) A9 promoter fragment of pWP78 is ligated into *Kpn*I, *Sma*I-cut pJIT60, forming pWP80 (Figure 9). This intermediate vector consists of a 936 bp A9 promoter fragment fused to a polylinker derived from pBluescript with a 35S CaMV polyadenylation signal. pJIT60 is identical to pJIT30 (Guerineau *et al.*, *Plant Mol. Biol.* **15**, 127-136 (1990)) except that the CaMV 35S promoter of pJIT30 is replaced by a double 35S CaMV promoter.

pWP83, an intermediate vector to express sense and anti-sense RNA using the constitutive CaMV 35S promoter, was constructed as follows. The A9 promoter of pWP80 is replaced by a 'double' CaMV 35S promoter by cloning the 785 bp *Kpn*I, *Xba*I fragment of pJIT60 into *Kpn*I, *Xba*I-cut pWP80, forming pWP83 (Figure 9).

pWP88, an intermediate vector to express sense and antisense RNA using the A3 promoter, was constructed as follows. The isolation of the *A. thaliana* tapetum-specific A3 promoter is described in WO-A-9211379. The CaMV promoter of pWP83 is replaced with the A3 promoter by cloning the 745 bp *Kpn*I, *Hin*dIII fragment of pWP87 (WO-A-9211379) into *Kpn*I, *Hin*dIII-cut pWP83, forming pWP88 (Figure 9).

pWP80, pWP83 and pWP88 are therefore identical apart from the promoter region and surrounding restriction enzyme sites.

Example 3B - Construction and expression in transgenic plants of chimeric gene fusion between the tapetum-specific A6 promoter and actinidin

The entire cDNA clone encoding actinidin is isolated as an *Eco*RI, *Bam*HI fragment from pK1W1450 (Podivinsky *et al*, *Nuc. Acids Res.* 17, 8363 (1989)) and is recloned into *Eco*RI, *Bam*HI-cut pBluescript KS- (Stratagene) forming pWP100. The oligonucleotide primers

5'GGGACTAGTCCATGGGTTTGCCCAAATCC 3' and
5' AATACGACTCACTATAG 3'

are used in a PCR reaction to generate a DNA fragment containing the entire coding region of actinidin, but with the sequence immediately before the initiating 'ATG' of the gene mutated to an *Spe*I site. The first primer is complementary to positions 38-55 bp of Figure 1 (Podivinsky *et al* 1989), and the second is homologous to a sequence immediately next to the *Kpn*I site of pBluescript KS-. This PCR fragment is digested with *Spe*I and *Sst*II and cloned into *Xba*I, *Sst*II-cut pDIH12 forming

pA6act (Figure 7B). The A6-actinidin chimeric gene is then recovered as a *Eco*RV fragment obtained by a partial *Eco*RV digest of pA6act and cloned into *Sma*I-cut pBin19 (Bevan *et al* 1984). The pBin19 derivative plasmid is transformed into *N. tabacum*, *B. napus* and *H. vulgare* where expression of actinidin in transgenic plants results in male sterility.

Examples 4 to 9 - Use of the coding sequence of the A6 gene to produce male sterile plants

Example 4A - Construction and expression in transgenic plants of chimeric gene fusion between the tapetum-specific promoter A9 and the A6 gene

The temporal pattern of expression of the tapetum-specific A3 and A9 genes determined from Northern analysis and promoter-GUS fusions show that both promoters are active at stages of anther development prior to the release of microspores from tetrads (see WO-A-9211379). Thus either promoter is suitable for driving the premature expression of $\beta(1,3)$ glucanase in anthers leading to male sterility (see discussion earlier in description). Chimeric fusions between these promoters and either the *B. napus* A6 cDNA or the *A. thaliana* A6 gene coding region can be constructed. In Figure 8a the construction of an A9 promoter fusion to the *A. thaliana* A6 gene is shown. Oligonucleotide primers are designed to the 5′ untranslated leader sequence of the *A. thaliana* gene and to the 3′ end of this gene such that a complete A6 gene can be obtained by use of the polymerase chain reaction from pDIH9. The primers are engineered with the restriction sites *Spe*I and *Sst*II for cloning the PCR A6 gene into vectors containing the tapetum-specific

promoters. The 5′ primer also contains a GTC sequence (underlined) which, in RNA, is a target for clevage by a ribozyme described in Example 5.

The 5′ oligonucleotide sequence is:-

5′ GGGACTAGTGTCACGCTGACAAAGACATGTCTCTTC 3′

The 3′ sequence is:-

5′ CCCCGCGGTCACAGAGTAACGCTCGGAAACTTGC 3′

The A6 PCR fragment is cloned as an 1548 bp *Spe*I, *Sst*II fragment into *Xba*I, *Sst*II-cut pWP80 (see WO-A-9211379), forming a transcriptional fusion between the A9 and A6 genes (Figure 8a). This construct is transferred to pBin19 as a *Sst*I, *Xho*I fragment.

Example 4B - Construction and expression in transgenic plants of chimeric gene fusions of the A9 or the A6 promoter to the A6 gene which lacks the sequences encoding the C-terminal extension of the anther-specific glucanase

The A6 protein has a long C-terminal extension when aligned against other previously sequenced plant glucanases (Figure 2). Extracellular glucanases do not have C-terminal extensions in contrast to those known to be located in the plant vacuole. The C-terminal extension in the anther-specific glucanase may thus be required for targeting to an intracellular storage body prior to its release into the locule. Removal of the C-terminal extension of A6 may lead to the immediate export of the glucanase into the locule, so that the A6 promoter in addition to the A3 and A9 promoters will cause male sterility when expressing such a construct. Figure 8b shows the construction of a chimeric genes between the A9

and A6 promoters and the anth r-specific glucanase that lacks the C-terminal extension. The oligonucleotide primers:-

5′ GGGACTAGTGTCACGCTGACAAAGACATGTCTCTTC 3′ and

5′ CCCCGCGGTTAGAAATCTACGTGTAGATTGG 3′

are used to PCR an 1208 bp fragment from pDIH9. This is either cloned as an *Spe*I, *Sst*II fragment into pWP80 or as an *Spe*I, *Sst*II fragment into pDIH12. Both chimeric genes are transferred to pBin19.

All the pBin19 constructs are transformed into *N. tabacum*, *B. napus* and *H. vulgare*. The transgenic plants are male-sterile.

Example 5 - Restoration of fertility of plants described in Example 4

Restoration of fertility is achieved by crossing the male sterile plants with transgenic plants that express in the tapetum a ribozyme that recognises and cleaves the sequence introduced into the 5′ leader of the PCR A6 gene (the natural A6 mRNA lacks this sequence and is not cleaved). Cleavage of the leader (3′ of the sequence GUC ie 14 bp 5′ of the ATG initiating codon of the A6 gene) removes the cap site of the PCR A6 transcript leading to rapid degradation of the PCR A6 mRNA and consequently a restoration of fertility in the F1 progeny.

Example 6 - Construction of chimeric genes producing sense and anti-sense RNA to the anther-specific glucanase in transgenic plants

The A3 and A9 promoters are transcriptionally active during the period that the anther-specific glucanase is expressed. Thus these promoters in addition to a

constitutive promoter (CaMV promoter) and the A6 promoter can be used to express anti-sense and sense RNA to the anther-specific glucanase. As described above, and in WO-A-9211379, the cDNAs isolated from the anther library have terminal adapters that enable the cDNA to be recovered as a *Not*I fragment. Thus the *B. napus* cDNA is digested with *Not*I and cloned in both orientations into the *Not*I sites of pWP80, pWP83, pWP88 (see WO-A-9211379 for these three plasmids) and pDIH12 (Figure 7). These chimeric genes are transferred to pBin19 and transformed into *N. tabacum*, *B. napus*, *H. vulgare* and *Z. mays*. The transgenic plants are male-sterile. Cytological examinations of male sterile plants expressing anti-sense A6 RNA, showed that the release of microspores from the tetrads, which requires the degredation of callose, is delayed compared to wild-type plants or is completely absent. Biochemically, these male sterile plants have reduced or undetectable callase levels in the locule fluid of the anther. Both observations confirm that A6 encodes callase.

Example 7 - Restoration of fertility of the transgenic plants expressing anti-sense A6 RNA, described in Example 6

Restoration of fertility is achieved in two ways. First the male sterile plants are crossed with plants containing additional copies of the *A. thaliana* A6 gene (the 3.2 kb *Eco*RI fragment of pDIH9 is transferred to pBin19 and this construct transformed into plants). The additional gene copies overcome the down-regulation of the callase product induced by the expression of antisense A6 RNA, resulting in male fertile F1 progeny. Secondly, restoration of fertility in plants expressing

antisense A6 RNA is achi ved by crossing these plants with plants hom zygous for chimeric gen fusi ns between a tapetum-specific promoter eg A3, A6 or A9 and a ribozyme directed against a GUC sequence within the antisense A6 RNA transcript at position 787-790 bp (Figure 1) for example. In the F1 progeny cleavage of the antisense A6 transcript results in destabilisation of the antisense RNA and a consequent restoration of fertility.

Example 8 - Expression of ribozymes, directed against the callase transcript, in transgenic plants

Comparison of the nucleotide sequences of the *B. napus* A6 cDNA and the *A. thaliana* A6 genomic sequence (Figure 5a) reveals 12 GUC trinucleotides that are shared by both sequences and are potential ribozyme target sequences. Two ribozymes are inserted into the *B. napus* A6 cDNA sequence by site-directed mutagenesis. Single stranded DNA from the plasmid A6, which contains the A6 cDNA cloned into the *Eco*RI site of BLUESRIPT™ SK⁻ (Stratagene), is annealed to the phosphorylated oligonucleotide primers shown below:-

5' CGGCGTCGTAGAGCTTCTGAAGATGGCCCGGTAGGGCCGAAACATGACCGGC 3' and

5' CGTTGGCTCCTTCCTGAAGATGGCCCGGTAGGGCCGAAACCGGTACGCACC 3'

The first primer encodes a ribozyme that is targeted to cleave the GUC at position 102 bp in figure 1 and the second the GUC at position 1169 bp. The underlined portion of the primers encodes the ribozyme.

After annealing, the second DNA strand is completed with nucleotides and Klenow. A plasmid with both ribozyme inserts, detected by duplicate colony hybridizations using the ribozyme primers (end-labelled) as probes, is cloned as a *Not*I fragment, in the anti-sense orientation,

into pWP80, pWP83, pWP88 and pDIH12. The chimeric A3, CaMV 35S, A9 and A6 promot r-callase ribozyme genes are transferred to pBin19 as described for the plasmids in Example 6. The chimeric genes are transferred to pBin19 and transformed into *N. tabacum*, *B. napus* and *H. vulgare*. The transgenic plants are male-sterile.

Example 9 - Restoration of fertility of the plants described in Example 8

Crossing the male sterile plants with homozygous transgenic plants expressing (from the A3, A6, A9 or CaMV 35S promoters) a ribozyme that cleaves a GUC sequence in the callase(mRNA)-specific ribozyme, results in progeny that are male fertile. The target GUC sequence for the restorer-ribozyme is located such that cleavage destabilises the target mRNA by either the removal of the CAP or the polyadenylation signal. This rapidly reduces the concentration of callase(mRNA)-specific ribozyme in the cytoplasm and results in fertility restoration. This restorer-ribozyme is constructed from plasmid A6 in a similar way to that described in Example 8.

CLAIMS

1. Recombinant or isolated DNA encoding a callase enzyme.

2. DNA as claimed in claim 1, wherein the enzyme has the activity of a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

3. DNA as claimed in claim 2, encoding a 53 kDa callase enzyme from *B. napus* or the equivalent enzyme from *Arabidopsis thaliana*.

4. DNA as claimed in claim 2 and having at least part of the sequence are shown in Figure 1 or Figure 4.

5. DNA as claimed in any one of claims 1 to 4 wherein the coding sequence is operatively linked to a promoter.

6. DNA as claimed in claim 5, wherein the promoter is tapetum-specific.

7. DNA as claimed in claim 6, wherein the promoter is a *Brassicaceae* A3 or A9 promoter.

8. Recombinant or isolated DNA comprising a promoter which naturally drives expression of a gene encoding a callase enzyme.

9. DNA as claimed in claim 8, wherein the callase enzyme is a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

10. DNA as claimed in claim 9 comprising a promoter which drives expression of a 53 kDa callase enzyme from *B. napus* or the equivalent enzyme from *Arabidopsis thaliana*.

11. DNA as claimed in claim 9, including a promoter sequence naturally 5′ to the coding region of the sequence shown in Figure 1.

12. DNA as claimed in claim 9, including the promoter 5′ to the coding region of the sequence shown in Figure 4.

13. DNA as claimed in any one of claims 8 to 12, wherein the promoter is operatively linked to DNA which, when expressed, causes male sterility in a plant.

14. DNA as claimed in claim 13, wherein the male sterility DNA encodes a lytic enzyme.

15. DNA as claimed in claim 14, wherein the lytic enzyme causes lysis of nucleic acid, protein, carbohydrate or lipid.

16. DNA as claimed in claim 15, wherein the lytic enzyme is a ribonuclease or a deoxyribonuclease.

17. DNA as claimed in claim 14, wherein the lytic enzyme causes lysis of a carbohydrate.

18. DNA as claimed in claim 17 wherein the lytic enzyme is a glucanase.

19. DNA as claimed in claim 18 including a signal sequence in a translational fusion with the glucanase coding sequence.

20. DNA as claimed in claim 15, wherein the lytic enzyme causes lysis f a prot in.

21. DNA as claimed in claim 20, wherein the proteolytic enzyme is actinidin or papain.

22. DNA as claimed in claim 13, wherein the male sterility DNA codes for RNA which is antisense to RNA normally found in a plant tapetum cell.

23. DNA as claimed in claim 22, wherein the male sterility DNA codes for RNA which is antisense to RNA encoding a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

24. Antisense nucleic acid which includes a transcribable strand of DNA complementary to at least part of the strand of DNA that is naturally transcribed in a gene encoding a callase enzyme.

25. Antisense nucleic acid as claimed in claim 24 wherein the callase enzyme is a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

26. Antisense nucleic acid as claimed in claim 24 or 25 wherein transcription is under the control of a constitutive promoter.

27. Antisense nucleic acid as claimed in claim 24 or 25 wherein transcription is under the control of a tapetum-specific promoter.

28. DNA as claimed in claim 13, wherein the male sterility DNA codes for RNA which is capable of cleavage of RNA normally found in a plant tapetum cell.

29. DNA as claimed in claim 28, wherein the male sterility DNA codes for RNA which is capable of cleavage of RNA encoding a callase enzyme, such as a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

30. DNA encoding a ribozyme capable of specific cleavage of RNA encoded by a callase gene.

31. Ribozyme-encoding DNA as claimed in claim 30, wherein the callase gene encodes a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

32. Ribozyme-encoding DNA as claimed in claim 30 or 31 wherein transcription is under the control of a constitutive promoter.

33. Ribozyme-encoding DNA as claimed in claim 30 or 31 wherein transcription is under the control of a tapetum-specific promoter.

34. DNA capable of specifically disrupting the proper expression of a callase gene.

35. DNA as claimed in claim 34, wherein the callase gene encodes a 53 kDa callase enzyme of *Brassica napus* or an equivalent protein in another member of the family *Brassicaceae*.

36. DNA as claimed in any one of claims 1 to 35 comprising a 3′ transcription regulation sequence.

37. DNA as claimed in claim 36, wherein the 3′ transcription regulation signals are derived from the Cauliflower Mosaic Virus 35S gene.

38. DNA as claimed in any one of claims 1 to 37 which is recombinant and which in the form of a vector.

39. DNA as claimed in claim 38, wherein the vector is a cloning vector and comprises one or more selectable markers.

40. A microbial host cell transfected or transformed with a vector as claimed in claim 38 or 39.

41. DNA as claimed in any one of claims 1 to 39, which includes a marker sequence which enables a plant transformed with the DNA to be distinguished from plants not so transformed.

42. DNA as claimed in claim 41, wherein the marker sequence confers antibiotic or herbicide resistance or codes for glucuronidase.

43. DNA as claimed in claim 41 or 42, wherein the marker sequence is under the control of a second promoter, which is not tapetum-specific.

44. DNA as claimed in claim 43, wherein the second promoter is derived from the Cauliflower Mosaic Virus (CaMV) 35S gene.

45. A plant cell including DNA as claimed in any one of claims 1 to 39 and 41 to 44.

46. A plant or part of a plant at least some of whose cells are as claimed in claim 45.

47. Propagating material from a plant as claimed in claim 46.

FIG. 1 (1/6)

DNA sequence of Brassica napus cDNA A6 and the deduced protein sequence of the ORF within A6.

```
   F  F  L  F  T  L  V  V  F  S  S  T  S  C  S  A  V  G  F
CTTTCTTCCTCTTCACCCTCGTCGTCTTTTCAAGTACAAGTTGCTCAGCGGTTGGGTTCC
        10         20         30         40         50         60

Q  H  P  H  R  Y  I  Q  K  K  T  M  L  E  L  A  S  K  I  G
AACATCCGCACAGGTATATACAGAAAAAAACGATGCTAGAGTTAGCCAGCAAGATTGGTA
        70         80         90        100        110        120

I  N  Y  G  R  Q  G  N  N  L  P  S  P  Y  Q  S  I  N  F  I
TTAACTATGGTAGACAAGGAAACAACCTACCATCTCCTTACCAATCGATCAATTTCATCA
       130        140        150        160        170        180

K  L  I  K  A  G  H  V  K  L  Y  D  A  D  P  E  S  L  T  L
AACTCATCAAAGCCGGTCATGTCAAGCTCTACGACGCCGATCCAGAGAGTCTAACACTCC
       190        200        210        220        230        240
```

FIG. 1 (2/6)

```
L  S  Q  T  N  L  Y  Y  V  T  I  A  V  P  T  H  Q  I  T  S  L
TCTCTCAACCCAATCTCTACGTCACCATAGCGGTGCCAACCCACCAGATCACTTCCCTCA
      250        260        270        280        290        300

S  A  N  Q  T  T  A  E  D  W  V  K  T  N  I  L  P  Y  Y  P
GCGCCAACCCAAACTACAGCTGAAGATTGGGTCAAAACCAATATCCTCCCTTACTACCCAC
      310        320        330        340        350        360

Q  T  Q  I  R  F  V  L  V  G  N  E  I  L  S  V  K  D  R  N
AAACACAAATACGATTTGTCCTTGTTGGAAACGAAATCCTCTCCGTCAAAGATAGGAACA
      370        380        390        400        410        420

I  T  G  N  V  V  P  A  M  R  K  I  V  N  S  L  R  A  H  G
TAACCGGCAATGTCGTACCGGCAATGCGAAAAATCGTGAACTCTCTCAGAGCCCATGGGA
      430        440        450        460        470        480

I  H  N  I  K  V  G  T  P  L  A  M  D  S  L  R  S  T  F  P
TTCACACATCAAAGTCGGTACACCTTTAGCTATGGATTCTCTTCGATCAACGTTTCCGC
      490        500        510        520        530        540
```

FIG. 1 (3/6)

```
P  S  N  S  T  F  R  G  D  I  A  L  P  L  M  L  P  L  L  K
CGTCGAACTCAACATTCCGGGGAGATATCGCCTTACCGTTAATGTTGCCGTTGCTGAAGT
        550        560        570        580        590        600

F  L  N  G  T  N  S  Y  F  F  I  N  L  Q  P  Y  F  R  W  S
TTCTCAACGGAACAAACTCTTACTTCTTTATCAATCTTCAACCTTACTTCCGTTGGTCAA
        610        620        630        640        650        660

R  N  P  N  H  T  T  L  D  F  A  L  F  Q  G  N  S  T  Y  T
GAAACCCTAATCACACCACGTTGGATTTCGCTCTGTTTCAAGGAAACTCAACTTATACCG
        670        680        690        700        710        720

D  P  H  T  G  L  V  Y  H  N  L  V  D  Q  M  L  D  S  V  I
ATCCTCATACCGGTTTGGTTTACCATAATCTTGTAGACCAAATGTTGGATTCGGTTATCT
        730        740        750        760        770        780

F  A  M  T  K  L  G  Y  P  Y  I  R  I  A  I  S  E  T  G  W
TCGCCATGACCAAGCTCGGTTATCCATACATCCGTATCGCAATCTCTGAAACCGGATGGC
        790        800        810        820        830        840
```

FIG. 1 (4/6)

```
P  N  S  G  D  I  D  E  I  G  A  N  V  F  N  A  A  T  Y  N
CTAACTCCGGCGACATCGACGAAATCGGAGCTAACGTTTTCAACGCCGCCACGTATAACC
      850        860        870        880        890        900

R  N  L  I  K  K  M  T  A  T  P  P  I  G  T  P  A  R  P  G
GGAATTTGATCAAGAAGATGACCGCAACTCCACCAATCGGTACACCAGCTAGACCCGGTT
      910        920        930        940        950        960

S  P  I  P  T  F  V  F  S  L  F  N  E  N  K  K  P  G  S  G
CACCTATACCGACATTTGTTTTCTCCTTATTTAACGAAAACAAGAAACCCGGTTCGGGAA
      970        980        990       1000       1010       1020

T  Q  R  H  W  G  I  L  H  P  D  G  T  P  I  Y  D  I  D  F
CACAAAGACATTGGGGAATCTTGCATCCGGACGGTACACCAATCTACGACATTGATTTTA
     1030       1040       1050       1060       1070       1080

T  G  Q  K  P  L  T  G  F  N  P  L  P  K  P  T  N  N  V  P
CCGGTCAAAAACCCTTAACCGGTTTTAACCCTCTGCCTAAACCGACGAATAACGTTCCTT
     1090       1100       1110       1120       1130       1140
```

FIG. 1 (5/6)

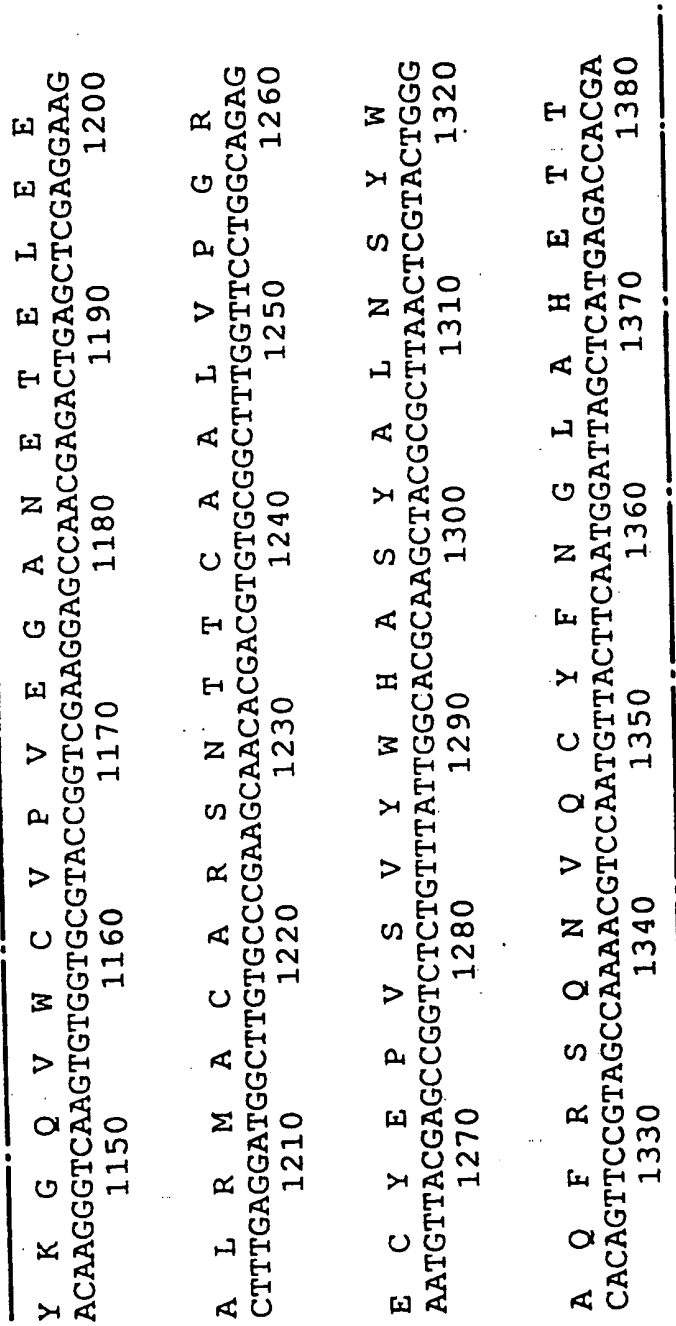

Y K G Q V W C V P V E G A N E T E L E E
ACAAGGGTCAAGTGTGGTGCGTACCGGTCGAAGGAGCCAACGAGACTGAGCTCGAGGAAG
1150 1160 1170 1180 1190 1200
A L R M A C A R S N T T C A A L V P G R
CTTTGAGGATGGCTTGTGCCCGAAGCAACACGACGTGTGCGGCTTTGGTTCCTGGCAGAG
1210 1220 1230 1240 1250 1260
E C Y E P V S V Y W H A S Y A L N S Y W
AATGTTACGAGCCGGTCTCTGTTTATTGGCACGCAAGCTACGCGCTTAACTCGTACTGGG
1270 1280 1290 1300 1310 1320
A Q F R S Q N V Q C Y F N G L A H E T T
CACAGTTCCGTAGCCAAAACGTCCAATGTTACTTCAATGGATTAGCTCATGAGACCACGA
1330 1340 1350 1360 1370 1380

FIG. 1 (6/6)

```
T  N  P  G  N  D  R  C  K  F  P  S  V  T  L  *
CTAACCCTGGAAATGATCGCTGCAAGTTCCGAGCGTTACTCTGTGAGGAAGAACGCCTG
     1390       1400       1410       1420       1430       1440

AAAGAGATTTAAGATGATCAAAGCTGGATTATTCGTATTTACTCATTCTAGATTTTCTGG
     1450       1460       1470       1480       1490       1500

TTTCTGTTTCGTGTGGCCTAATGTTGAGAAAA
     1510       1520       1530
```

FIG. 2 (1/5)

Alignment of the deduced primary structures of the *B.napus* and the *A.thaliana* A6 genes with the primary structure of previously described glucanases.

```
* :=>  match across all seqs.
. :=>  conservative substitutions

NPGLUC                                    ALQMAAIILLGLLVSSTEIVGAQSVGVCYGMLGNNLPPASQV
BEAN                                                      QIGVCYGMMGNNLPSANEV
PR-Q                                  QFLFSLQMAHLIVTLLLLSVLTLATLDFTGAQAGVCYGRQGNGLPSPADV
BARLEY                                  MARKDVASMFAAALFIGAFAAVPTSVQSIGVCYGVIGNNLPSRSDV
At G62                          MSLLAFFLFTILVFSSSCCSATRFQ-GHRYMQRKTMLDLASKIGINYGRRGNNLPSPYQS
Bn A6                                FFLFTLVVFSSTSCSAVGFQHPHRYIQKKTMLELASKIGINYGRQGNNLPSPYQS
                                                          *. **  **.**.  .

NPGLUC                          VQLYKSKNIRRMRLYDPNQAALQALRGSNIEVMLGVPNSDLQNIAANPSNANNWVQRNVR
BEAN                            INLYRSNNIRRMRLYDPNGAALGALRNSGIELILGVPNSDLQGLATNADTARQWVQRNVL
PR-Q                            VSLCNRNNIRRMRIYDPDQPTLEALRGSNIELMLGVPNPDLENVAASQANADTWVQNNVR
BARLEY                          VQLYRSKGINGMRIYFADGQALSALRNSGIGLILDIGNDQLANIAASTSNAASWVQNNVR
At G62                          INFIKSIKAGHVKLYDADPESLTLLSQTNLYVTITVPNHQITALSSNQTIADEWVRTNIL
Bn A6                           INFIKLIKAGHVKLYDADPESLTLLSQTNLYVTIAVPTHQITSLSANQTTAEDWVKTNIL
                                . . .    ...* ..  .*  *. ... . ... . .. ..... . *  **. *.
```

FIG. 2 (2/5)

```
NPGLUC      NFWPAVKFRYIAVGNEVSPVTGTSSLTRYLLPAMRNIRNAISSAGLQNNIKVSSSVDMTL
BEAN        NFWPSVKIKYIAVGNEVSPVGGSSWYAQYVLPAVQNVYGAVRAQGLHDGIKVSTAIDMTL
PR-Q        NY-GNVKFRYIAVGNEVSPLNENSKYVPVLLNAMRNIQTAISGAGLGNQIKVSTAIETGL
BARLEY      PYYPAVNIKYIAAGNEVQGGA-----TQSILPAMRNLNAALSAAGLG-AIKVSTSIRFDE
At G62      PYYPQTQIRFVLVGNEILSYNSGN-VSVNLVPAMRKIVNSLRLHGIHN-IKVGTPLAMDS
Bn A6       PYYPQTQIRFVLVGNEILSVKDRN-ITGNVVPAMRKIVNSLRAHGIHN-IKVGTPLAMDS
             .   ..... .***.           .. *.... .... *.   ***....  .

NPGLUC      IGNSFPPSQGSFRNDVR-SFIDPIIGFVRRINSPLLVNIYPYFSYAGNPRDISLPYALFT
BEAN        IGNSYPPSQGSFRGDVR-SYLDPIIGYLLYASAPLHVNVYPYFSYSGNPRDISLPYALFT
PR-Q        TTDTSPPSNGRFKDDVR-QFIEPIINFLVTNRAPLLVNLYPYFAIANNA-DIKLEYALFT
BARLEY      VANSFPPSAGVFKN----AYMTDVARLLASTGAPLLANVYPYFAYRDNPGSISLNYATFQ
At G62      LRSSFPRSNGTFREEITGPVMLPLLKFLNGTNSYFFLNVHPYFRWSRNPMNTSLDFALFQ
Bn A6       LRSTFPPSNSTFRGDIALPLMLPLLKFLNGTNSYFFINLQPYFRWSRNPNHTTLDFALFQ
              .. *.*.. *..     .  .  .    . .  *. ***    *.  ..* .* *
```

FIG. 2 (3/5)

```
NPGLUC      APNVVV-QDGSLGYRNLFDAMSDAVYAALSRAGGGSIEIVVSESGWPSAGAFA-ATTN--
BEAN        SPNVVV-RDGQYGYQNLFDAMLDSVHAAIDNTRIGYVEVVVSESGWPSDGGFG-ATYD--
PR-Q        SSEVVV-NDNGRGYRNLFDAILDATYSALEKASGSSLEIVVSESGWPSAGAGQLTSID--
BARLEY      PGTTVRDQNNGLTYTSLFDAMVDAVYAALEKAGAPAVKVVVSESGWPSAGGFA-ASAG--
At G62      GHSTYTDPQTGLVYRNLLDQMLDSVLFAMTKLGYPHMRLAISETGWPNFGDIDETGANIL
Bn A6       GNSTYTDPHTGLVYHNLVDQMLDSVIFAMTKLGYPYIRIAISETGWPNSGDIDEIGANVF
              ..    ..   * .* *.. *..  *...     . ...**.***. *.    . .

NPGLUC      NAATYYKNLIQHVKR----GSPRRPNKVIETYLFAMFDENNKNPE-LEKHFGLFSPNKQP
BEAN        NARVYLDNLVRRAGR----GSPRRPSKPTETYIFAMFDENQKSPE-IEKHFGLFKPSKEK
PR-Q        NARTYNNNLISHVKG----GSPKRPSGPIETYVFALFDEDQKDPE-IEKHFGLFSANMQP
BARLEY      NARTYNQGLINHVGG----GTPKKRE-ALETYIFAMFNENQKTGDATERSFGLFNPDKSP
At G62      NAATYNRNLIKKMSASPPIGTPSRPGLPIPTFVFSLFNENQKSGSGTQRHWGIFDPDGSP
Bn A6       NAATYNRNLIKKMTATPPIGTPARPGSPIPTFVFSLFNENKKPGSGTQRHWGILHPDGTP
            ** .*  .*. .       *.* ...    *..*..*.*..*  .  .. .*.. ..
```

FIG. 2 (4/5)

```
NPGLUC      KYPLSFGFS--DRYWDISAENNATAASLISEM
BEAN        KYPFGFGAQRMQRLLLMSSMQHIPLRVTCKLEPSSQSLL
PR-Q        KYQISFN
BARLEY      AYNIQF
At G62      IYDVDFTGQTPLTGFNPLPKPTNNVPYKGQVWCVPVEGANETELEETLRMACAQSNTTCA
Bn A6       IYDIDFTGQKPLTGFNPLPKPTNNVPYKGQVWCVPVEGANETELEEALRMACARSNTTCA
             *    *

At G6       ALAPGRECYEPVSIYWHASYALNSYWAQFRNQSIQCFFNGLAHETTTNPGNDRCKFP
Bn A6       ALVPGRECYEPVSVYWHASYALNSYWAQFRSQNVQCYFNGLAHETTTNPGNDRCKFP


At G62      SVTL
Bn A6       SVTL
```

FIG. 2 (5/5)

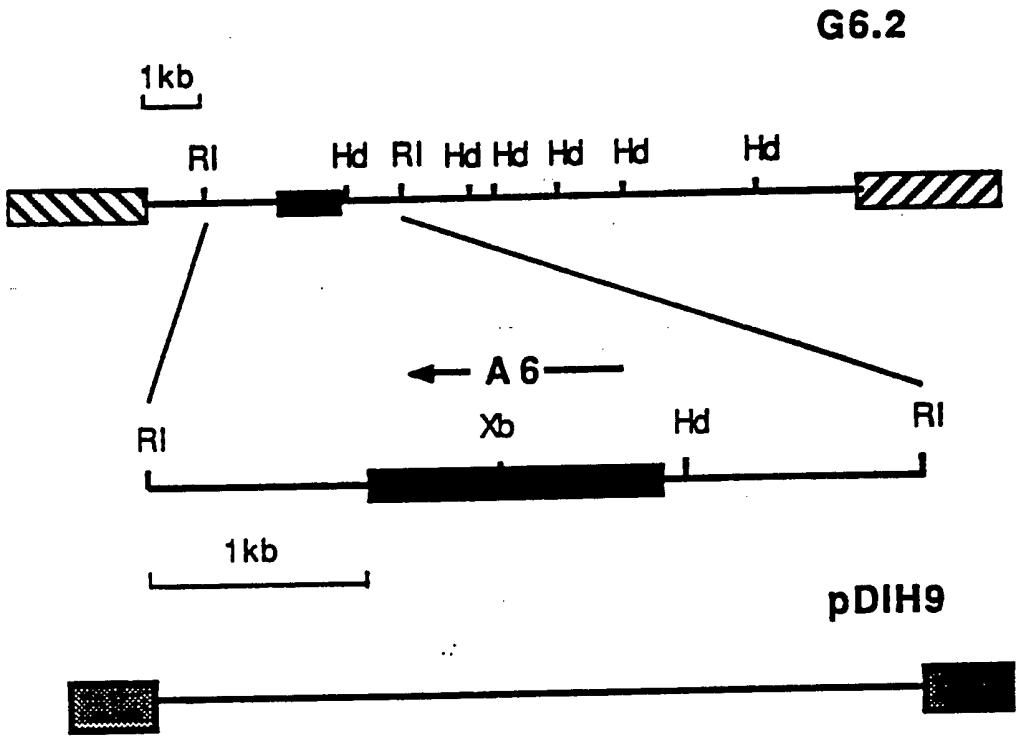
NPGLUC- Tobacco B 1,3-glucanase (Basic):-
De Loose M., Alliotte T., Gheysen G., Genetello C., Gielen J., Soetaert P., Van Montagu M. and Inz D. (1988) Gene 70, 13-23

BEAN - Bean B 1,3-glucanase:-
Edington, B.V., Lamb, C.J. and Dixon, R.A. (1991) Plant Mol. Biol. 16, 81-94

PR-Q - Tobacco B 1,3-glucanase (Extra-cellular):-
Payne, G., Ward, E., Gaffney, T., Ahl Goy, P., Moyer, M., Harper, A., Meins, F.Jr. and Ryals, J. (1990) Plant Mol. Biol. 15, 797-808.

BARLEY- Barley B 1,3-glucanase:-
Hoj, P.B., Hartman, D.J., Morrice, N.A., Doan, D.N.P. and Fincher, G.B. (1989) Plant Mol. Biol. 13, 31-42.

FIG. 3



Key to DNA sequences

- Arabidopsis
- lambda Dash
- pTZ18 vector
- A6 coding region

FIG. 4 (1/9)

DNA sequence of the *A.thaliana* A6 gene together with the deduced primary structure of A6.

```
GAATTCACACAAAGCAATTAACAAAGTTAACCAAATCCCAAATTCGAATTTGGTTCCCTA
        10        20        30        40        50        60

TTCTACAGCCTAACCGTATTCTGAGATCTGTAACAGAGTCATGAACAGAAAATACCAACC
        70        80        90       100       110       120

TCGAGCTGACCGGAGCGGCACGATTTTGACTCGTCGAGCGTGTAAAAGAAGGAAGTACCA
       130       140       150       160       170       180

TTGTTCCATTCAAGGTCGTAGGTAATACCACCGAGCTGCTCCTGGATGATATTGAAATTA
       190       200       210       220       230       240
```

FIG. 4 (2/9)

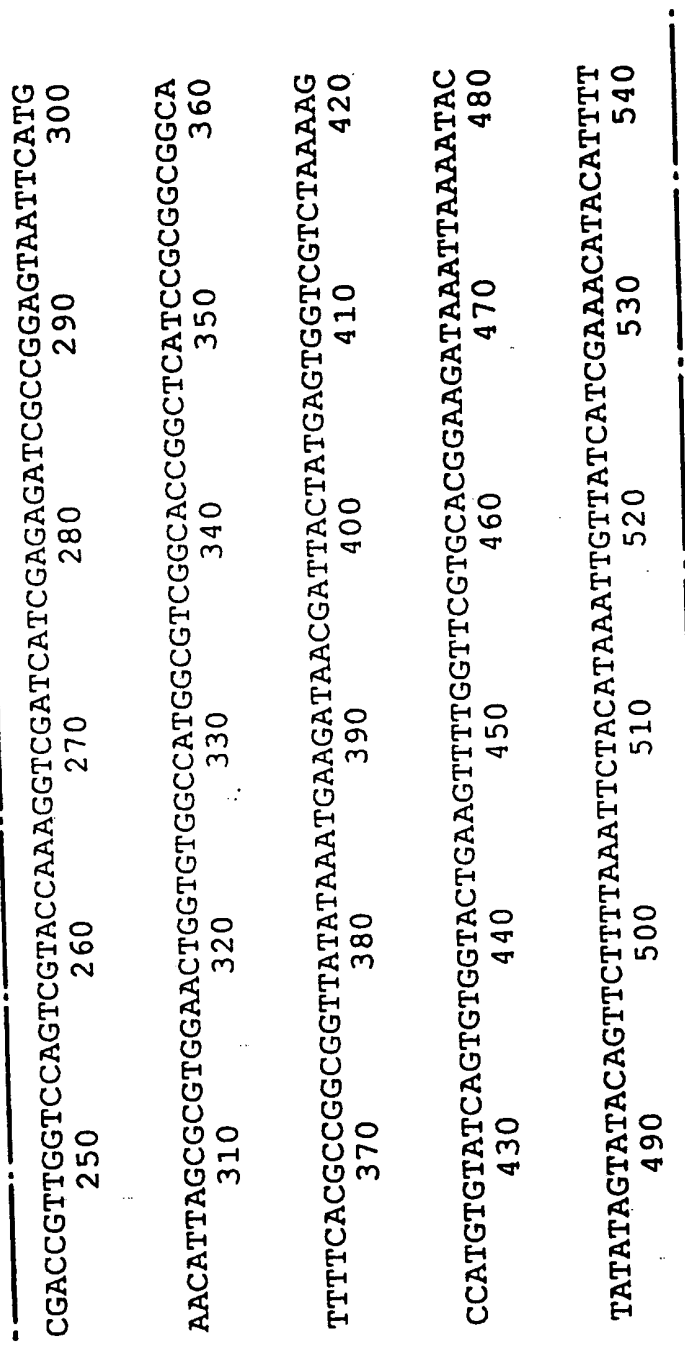

CGACCGTTGGTCCAGTCGTACCAAAGGTCGATCATCGAGAGATCGCCGGAGTAATTCATG
250 260 270 280 290 300
AACATTAGCGCGTGGAACTGGTGTGGCCATGGCGTCGGCACCGGCTCATCCGCGGCGGCA
310 320 330 340 350 360
TTTTCACGCCGGCGGTTATATAAATGAAGATAACGATTACTATGAGTGGTCGTCTAAAAG
370 380 390 400 410 420
CCATGTGTATCAGTGTGGTACTGAAGTTTTGGTTCGTGCACGGAAGATAAATTAAAATAC
430 440 450 460 470 480
TATATAGTATACAGTTCTTTTAAATTCTACATAAATTGTTATCATCGAAACATACATTTT
490 500 510 520 530 540

FIG. 4 (3/9)

```
                                                                    .
AGTCCATTAGTCTACTAAACTCATTATTGATGTATAATCTCTCAATCTACAATCAGAAAT
      550       560       570       580       590       600

                                                                    .
GTATTTGCAAAATTAACAATATTGGGGAAAGTGTTTCTTGGTTCAATTTGAACCGATCCA
      610       620       630       640       650       660

                                                                    .
ACCAACAATCCTTTTAAAATCATAGCACAAAAGAACTATGAGAGTTTCAAAAAGAAAATC
      670       680       690       700       710       720

                                                                    .
AAAAGCCAAAACAAAGCTTTTCTTGCATGACTCAATAAACCTACACTACACCATACTCTT
      730       740       750       760       770       780

                                                                    .
ACTTATAAACCTCATCTCCAATGCCACACCATTCCATCTTAAAATCACATTCTGATCATC
      790       800       810       820       830       840
```

FIG. 4 (4/9)

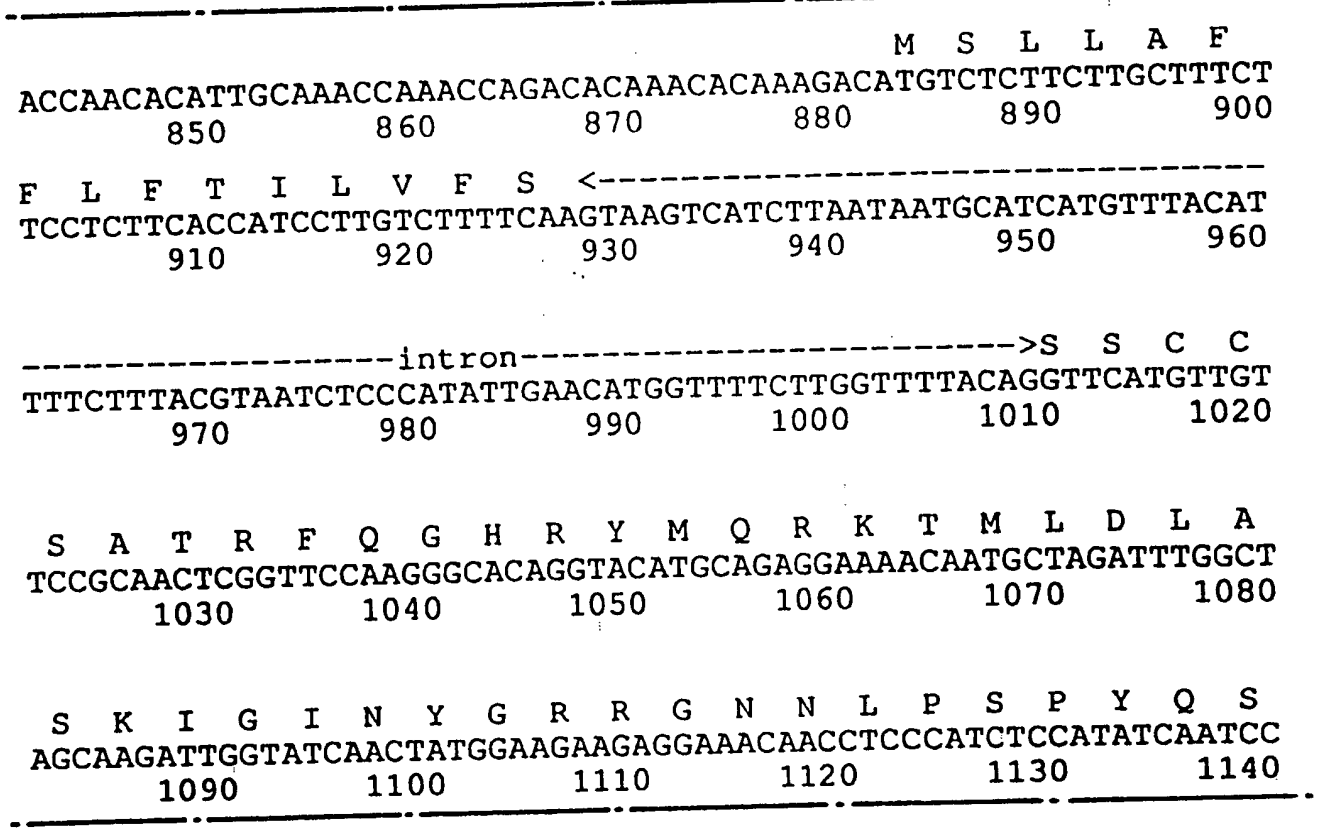

M S L L A F
ACCAACACATTGCAAACCAAACCAGACACAAACACAAAGACATGTCTCTTCTTGCTTTCT
850 860 870 880 890 900
F L F T I L V F S <----------
TCCTCTTCACCATCCTTGTCTTTTCAAGTAAGTCATCTTAATAATGCATCATGTTTACAT
910 920 930 940 950 960
----------intron----------->S S C C
TTTCTTTACGTAATCTCCCATATTGAACATGGTTTTCTTGGTTTTACAGGTTCATGTTGT
970 980 990 1000 1010 1020
S A T R F Q G H R Y M Q R K T M L D L A
TCCGCAACTCGGTTCCAAGGGCACAGGTACATGCAGAGGAAAACAATGCTAGATTTGGCT
1030 1040 1050 1060 1070 1080
S K I G I N Y G R R G N N L P S P Y Q S
AGCAAGATTGGTATCAACTATGGAAGAAGAGGAAACAACCTCCCATCTCCATATCAATCC
1090 1100 1110 1120 1130 1140

FIG. 4 (5/9)

```
 I  N  F  I  K  S  I  K  A  G  H  V  K  L  Y  D  A  D  P  E
ATCAACTTCATCAAATCTATCAAAGCTGGTCATGTCAAGCTCTATGACGCCGATCCAGAG
      1150       1160       1170       1180       1190       1200

 S  L  T  L  L  S  Q  T  N  L  Y  V  T  I  T  V  P  N  H  Q
AGTCTCACACTCCTCTCTCAAACCAATCTCTACGTCACCATAACCGTCCCTAACCACCAA
      1210       1220       1230       1240       1250       1260

 I  T  A  L  S  S  N  Q  T  I  A  D  E  W  V  R  T  N  I  L
ATCACCGCCCTCAGCTCTAACCAAACCATAGCTGACGAATGGGTCAGAACTAACATCCTC
      1270       1280       1290       1300       1310       1320

 P  Y  Y  P  Q  T  Q  I  R  F  V  L  V  G  N  E  I  L  S  Y
CCTTACTATCCACAAACACAAATCCGTTTTGTCCTTGTCGGAAACGAAATCCTCAGCTAC
      1330       1340       1350       1360       1370       1380

 N  S  G  N  V  S  V  N  L  V  P  A  M  R  K  I  V  N  S  L
AATTCTGGGAATGTCTCTGTGAATCTTGTACCGGCGATGCGCAAGATCGTTAACTCACTC
      1390       1400       1410       1420       1430       1440
```

FIG. 4 (6/9)

```
R  L  H  G  I  H  N  N  I  K  V  G  G  T  P  P  L  A  M  D  S  L  R
AGATTACATGGGATTCACAACATCAAAGTTGGGACACCTCTAGCTATGGATTCTCTCCGG
      1450       1460       1470       1480       1490       1500

S  S  F  P  R  S  N  G  T  F  R  E  E  I  T  G  P  V  M  L
TCGTCGTTTCCTCGATCGAACGGAACATTCCGGGAAGAAATCACCGGACCGGTGATGTTA
      1510       1520       1530       1540       1550       1560

P  L  L  K  F  L  N  G  T  N  S  Y  F  F  L  N  V  H  P  Y
CCGTTGCTGAAGTTTCTCAACGGAACAAACTCTTACTTCTTCCTTAATGTTCATCCTTAC
      1570       1580       1590       1600       1610       1620

F  R  W  S  R  N  P  M  N  T  S  L  D  F  A  L  F  Q  G  H
TTCCGTTGGTCAAGAAACCCCATGAACACCAGTTTGGATTTTGCTCTGTTCCAAGGACAC
      1630       1640       1650       1660       1670       1680
```

FIG. 4 (7/9)

```
 S  T  Y  T  D  P  Q  T  G  L  V  Y  R  N  L  L  D  Q  M  L
TCAACCTATACCGATCCTCAAACCGGTTTGGTTTACCGTAATCTTCTAGACCAAATGTTG
      1690       1700       1710       1720       1730       1740

 D  S  V  L  F  A  M  T  K  L  G  Y  P  H  M  R  L  A  I  S
GATTCGGTTCTCTTCGCCATGACCAAACTCGGTTATCCACATATGCGCCTCGCGATCTCT
      1750       1760       1770       1780       1790       1800

 E  T  G  W  P  N  F  G  D  I  D  E  T  G  A  N  I  L  N  A
GAAACCGGATGGCCTAATTTCGGTGACATCGACGAAACCGGAGCCAACATTCTCAACGCA
      1810       1820       1830       1840       1850       1860

 A  T  Y  N  R  N  L  I  K  K  M  S  A  S  P  P  I  G  T  P
GCTACCTATAACCGTAATCTGATCAAGAAGATGAGCGCAAGTCCTCCAATCGGTACACCA
      1870       1880       1890       1900       1910       1920

 S  R  P  G  L  P  I  P  T  F  V  F  S  L  F  N  E  N  Q  K
TCAAGACCCGGTTTACCAATACCGACATTTGTTTTCTCCTTATTCAACGAAAACCAGAAA
      1930       1940       1950       1960       1970       1980

 S  G  S  G  T  Q  R  H  W  G  I  F  D  P  D  G  S  P  I  Y
TCCGGTTCGGGGACACAGAGACATTGGGGAATCTTCGATCCCGACGGTTCACCAATCTAC
      1990       2000       2010       2020       2030       2040
```

FIG. 4 (8/9)

```
D V D F T G Q T P L T G F N P L P K P T
GACGTAGATTTCACCGGTCAAACACCCTTAACCGGTTTCAACCCGTTACCTAAACCGACG
      2050      2060      2070      2080      2090      2100

N N V P Y K G Q V W C V P V E G A N E T
AACAACGTTCCTTACAAAGGTCAAGTGTGGTGCGTACCAGTCGAAGGAGCCAACGAGACT
      2110      2120      2130      2140      2150      2160

E L E E T L R M A C A Q S N T T C A A L
GAGCTTGAAGAAACATTGAGGATGGCTTGTGCCCAAGCAACACCACTTGTGCAGCTTTA
      2170      2180      2190      2200      2210      2220

A P G R E C Y E P V S I Y W H A S Y A L
GCTCCTGGGAGAGAATGTTACGAACCAGTCTCCATTTATTGGCATGCAAGCTACGCGCTT
      2230      2240      2250      2260      2270      2280

N S Y W A Q F R N Q S I Q C F F N G L A
AATTCGTACTGGGCTCAGTTTCGTAACCAAAGCATTCAATGTTTCTTCAATGGATTGGCT
      2290      2300      2310      2320      2330      2340
```

FIG.4 (9/9)

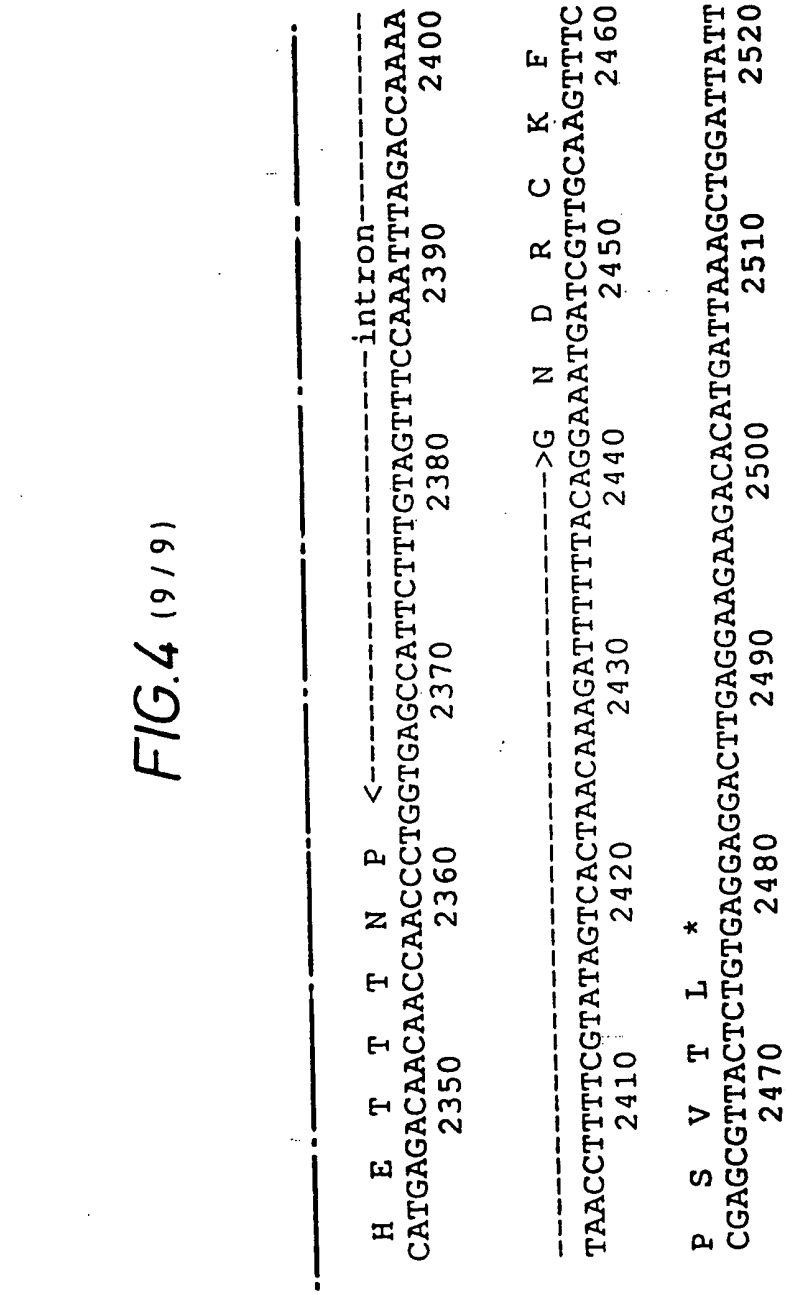

H E T T T N P <----------intron----------
CATGAGACAACAACCAACCCTGGTGAGCCATTCTTTGTAGTTTCCAAATTTAGACCAAAA
2350 2360 2370 2380 2390 2400
----------->G N D R C K F
TAACCTTTTCGTATAGTCACTAACAAAGATTTTTTACAGGAAATGATCGTTGCAAGTTTC
2410 2420 2430 2440 2450 2460
P S V T L *
CGAGCGTTACTCTGTGAGGAGGACTTGAGGAAGAGACACATGATTAAAGCTGGATTATT
2470 2480 2490 2500 2510 2520
CGTATAACTCAATAATGTTCCTTATCTTTTTTTTTATTATACCTTTTTT
2530 2540 2550 2560

FIG. 5a (1/8)

Alignment of the DNA sequences of the *A.thaliana* A6 gene and the *B.napus* A6 cDNA.

```
At   862 CCAGACACAAACACAAAGACATGTCTCTTCTTGCTTTCTTCCTCTTCACC 911
                                    ||||||||||||||||||
Bn     1 ..............................CTTTCTTCCTCTTCACC 17

     912 ATCCTTGTCTTTTCAAGTAAGTCATCTTAATAATGCATCATGTTTACATT 961
          || | ||||||||||
      18 CTCGTCGTCTTTTCAA..................................  40

     962 TTCTTTACGTAATCTCCCATATTGAACATGGTTTTCTTGGTTTTACAGGT 1011
                                                        ||
      35 ...........................................GT 35

    1012 TCATGTTGTTCCGCAACTCGGTTCCAA...GGGCACAGGTACATGCAGAG 1058
          || |||| || ||   | ||||||||     |||||||| || ||||
      36 ACAAGTTGCTCAGCGGTTGGGTTCCAACATCCGCACAGGTATATACAGAA 85
```

FIG. 5a (2/8)

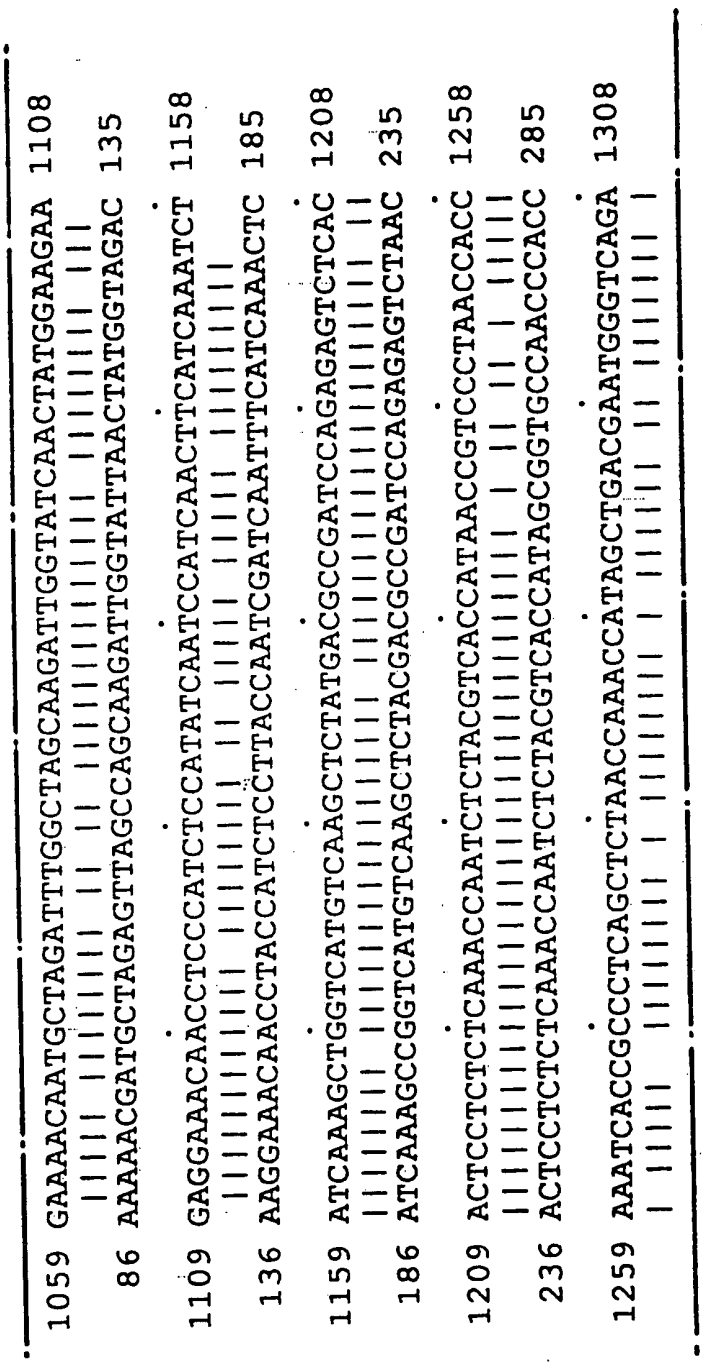

1059 GAAAACAATGCTAGATTTGGCTAGCAAGATTGGTATCAACTATGGAAGAA 1108
86 AAAAACGATGCTAGAGTTAGCCAGCAAGATTGGTATTAACTATGGTAGAC 135
1109 GAGGAAACAACCTCCCATCTCCATATCAATCCATCAACTTCATCAAATCT 1158
136 AAGGAAACAACCTACCATCTCCTTACCAATCGATCAATTTCATCAAACTC 185
1159 ATCAAAGCTGGTCATGTCAAGCTCTATGACGCCGATCCAGAGAGTCTCAC 1208
186 ATCAAAGCCGGTCATGTCAAGCTCTACGACGCCGATCCAGAGAGTCTAAC 235
1209 ACTCCTCTCTCAAACCAATCTCTACGTCACCATAACCGTCCCTAACCACC 1258
236 ACTCCTCTCTCAAACCAATCTCTACGTCACCATAGCGGTGCCAACCCACC 285
1259 AAATCACCGCCCTCAGCTCTAACCAAACCATAGCTGACGAATGGGTCAGA 1308

FIG. 5a (3/8)

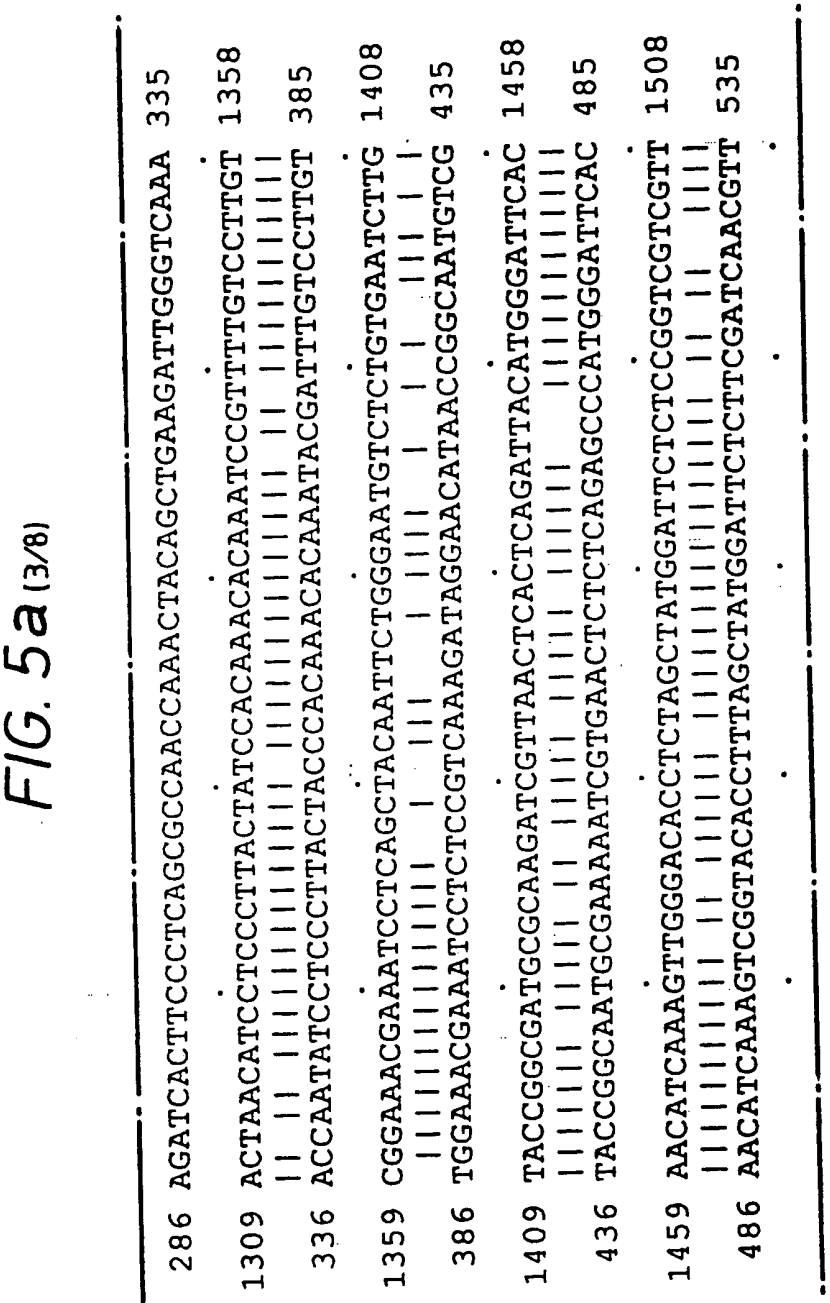

286 AGATCACTTCCCTCAGCGCCAACCAAACTACAGCTGAAGATTGGGTCAAA 335
1309 ACTAACATCCTCCCTTACTATCCACAAACACAAATCCGTTTTTGTCCTTGT 1358
336 ACCAATATCCTCCCTTACTACCCACAAACACAAATACGATTTGTCCTTGT 385
1359 CGGAAACGAAATCCTCAGCTACAATTCTGGGAATGTCTCTGTGAATCTTG 1408
386 TGGAAACGAAATCCTCTCCGTCAAAGATAGGAACATAACCGGCAATGTCG 435
1409 TACCGGCGATGCGCAAGATCGTTAACTCACTCAGATTACATGGGATTCAC 1458
436 TACCGGCAATGCGAAAAATCGTGAACTCTCTCAGAGCCCATGGGATTCAC 485
1459 AACATCAAAGTTGGGACACCTCTAGCTATGGATTCTCTCCGGTCGTCGTT 1508
486 AACATCAAAGTCGGTACACCTTTAGCTATGGATTCTCTTCGATCAACGTT 535

FIG. 5a (4/8)

```
1509 TCCTCGATCGAACGGAACATTCCGGGAAGAAATCACCGGACCGGTGATGT 1558
     ||| |  ||||||  |||||||||||| ||| ||| ||  |||| | ||||
 536 TCCGCCGTCGAACTCAACATTCCGGGGAGATATCGCCTTACCGTTAATGT 585

1559 TACCGTTGCTGAAGTTTCTCAACGGAACAAACTCTTACTTCTTCCTTAAT 1608
     | ||||||||||||||||||||||||||||||||||||||||  | |||
 586 TGCCGTTGCTGAAGTTTCTCAACGGAACAAACTCTTACTTCTTTATCAAT 635

1609 GTTCATCCTTACTTCCGTTGGTCAAGAAACCCCATGAACACCAGTTTGGA 1658
      |||| |||||||||||||||||||||||| |   ||||||  |||||
 636 CTTCAACCTTACTTCCGTTGGTCAAGAAACCCTAATCACACCACGTTGGA 685

1659 TTTTGCTCTGTTCCAAGGACACTCAACCTATACCGATCCTCAAACCGGTT 1708
     ||| |||||||| |||||| ||||||| |||||||||||||| |||||||
 686 TTTCGCTCTGTTTCAAGGAAACTCAACTTATACCGATCCTCATACCGGTT 735

1709 TGGTTTACCGTAATCTTCTAGACCAAATGTTGGATTCGGTTCTCTTCGCC 1758
     ||||||||| ||||||| |||||||||||||||||||| ||||||||
 736 TGGTTTACCATAATCTTGTAGACCAAATGTTGGATTCGGTTATCTTCGCC 785
```

FIG. 5a (5/8)

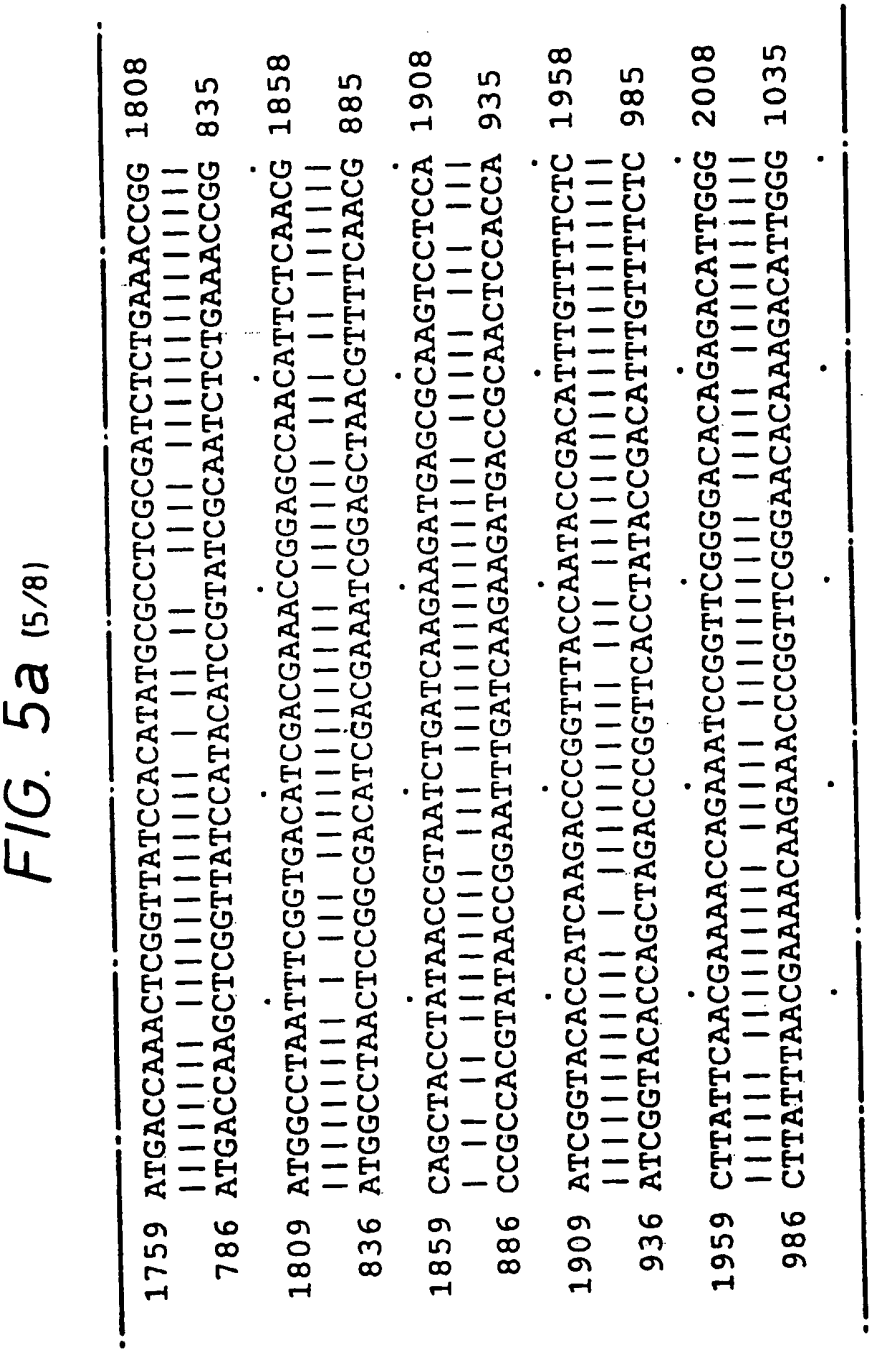

1759 ATGACCAAACTCGGTTATCCACATATGCGCCTCGCGATCTCTGAAACCGG 1808
786 ATGACCAAGCTCGGTTATCCATACATCCGTATCGCAATCTCTGAAACCGG 835
1809 ATGGCCTAATTTCGGTGACATCGACGAAACCGGAGCCAACATTCTCAACG 1858
836 ATGGCCTAACTCCGGCGACATCGACGAAATCGGAGCTAACGTTTTCAACG 885
1859 CAGCTACCTATAACCGTAATCTGATCAAGAAGATGAGCGCAAGTCCTCCA 1908
886 CCGCCACGTATAACCGGAATTTGATCAAGAAGATGACCGCAACTCCACCA 935
1909 ATCGGTACACCATCAAGACCCGGTTTACCAATACCGACATTTGTTTTCTC 1958
936 ATCGGTACACCAGCTAGACCCGGTTCACCTATACCGACATTTGTTTTCTC 985
1959 CTTATTCAACGAAAACCAGAAATCCGGTTCGGGGACACAGAGACATTGGG 2008
986 CTTATTTAACGAAAACAAGAAACCCGGTTCGGGAACACAAAGACATTGGG 1035

FIG. 5a (6/8)

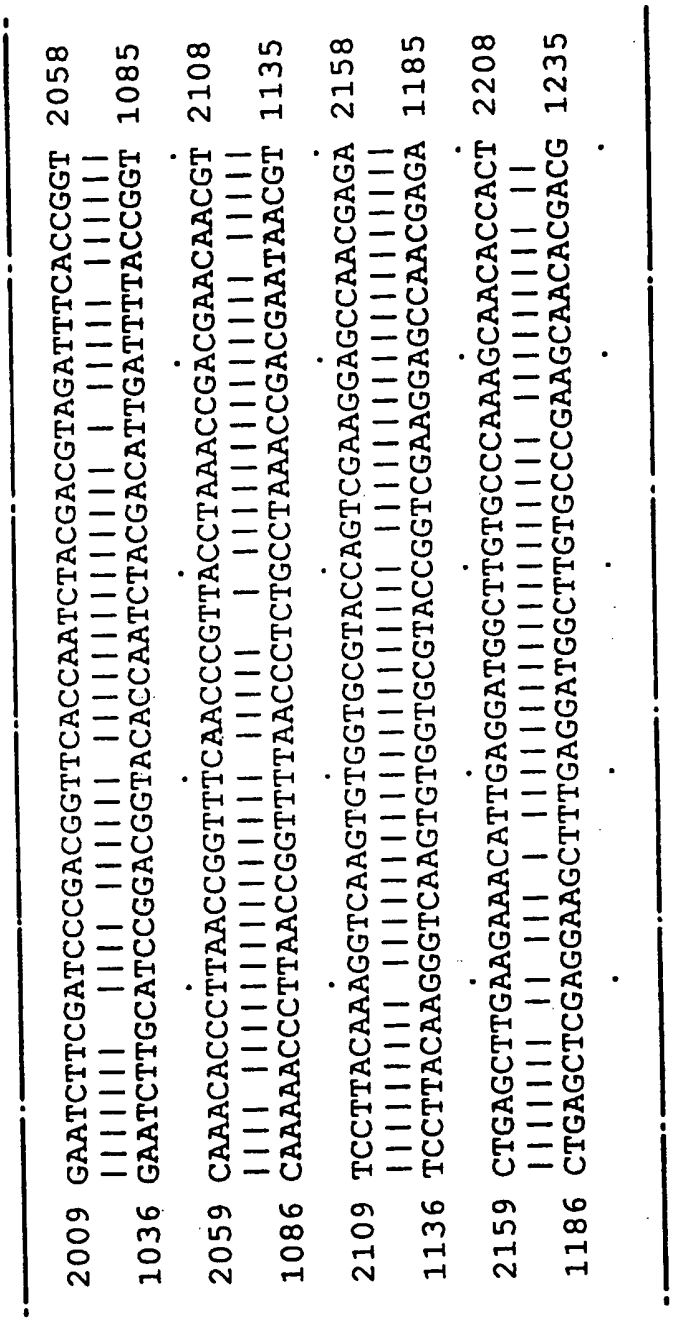

2009 GAATCTTCGATCCCGACGGTTCACCAATCTACGACGTAGATTTCACCGGT 2058
1036 GAATCTTGCATCCGGACGGTACACCAATCTACGACATTGATTTTACCGGT 1085
2059 CAAACACCCTTAACCGGTTTCAACCCGTTACCTAAACCGACGAACAACGT 2108
1086 CAAAAACCCTTAACCGGTTTTAACCCTCTGCCTAAACCGACGAATAACGT 1135
2109 TCCTTACAAAGGTCAAGTGTGGTGCGTACCAGTCGAAGGAGCCAACGAGA 2158
1136 TCCTTACAAGGGTCAAGTGTGGTGCGTACCGGTCGAAGGAGCCAACGAGA 1185
2159 CTGAGCTTGAAGAAACATTGAGGATGGCTTGTGCCCAAAGCAACACCACT 2208
1186 CTGAGCTCGAGGAAGCTTTGAGGATGGCTTGTGCCCGAAGCAACACGACG 1235

FIG. 5a (7/8)

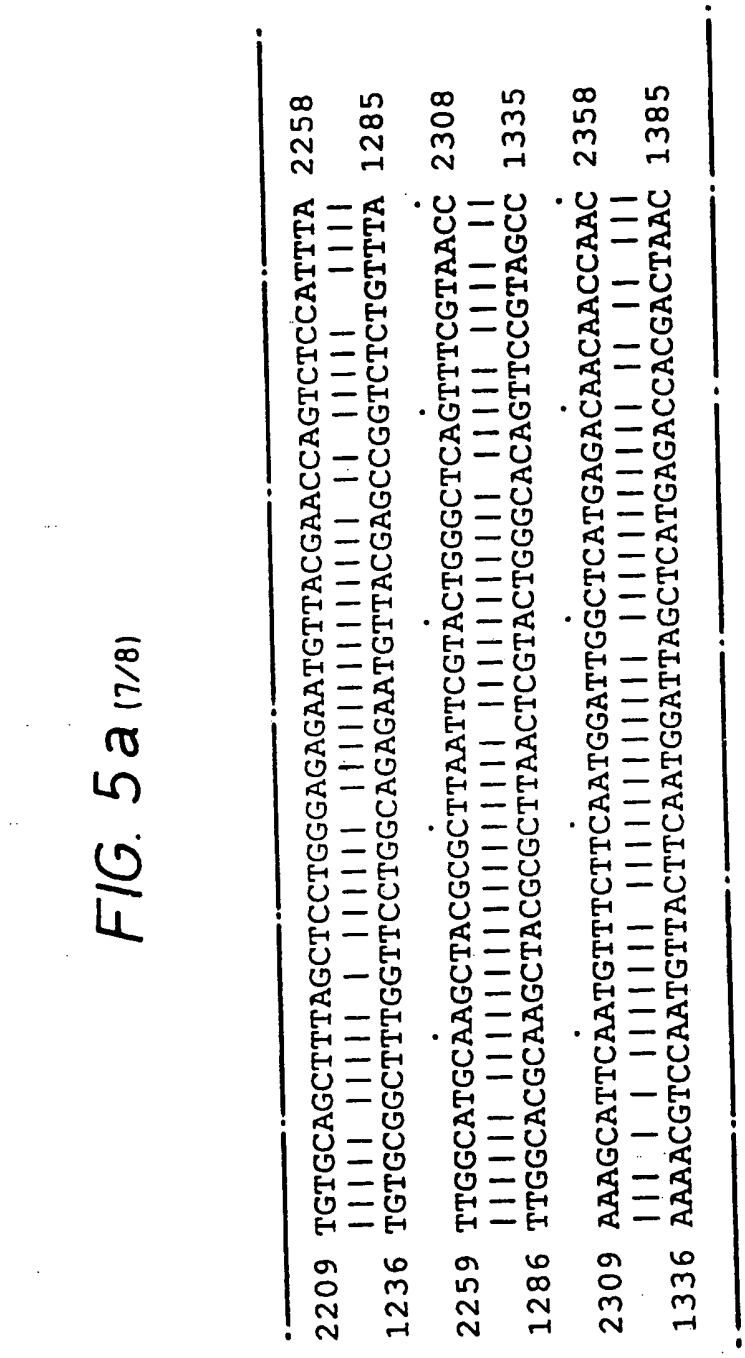

2209 TGTGCAGCTTTAGCTCCTGGGAGAGAATGTTACGAACCAGTCTCCATTTA 2258
1236 TGTGCGGCTTTGGTTCCTGGCAGAGAATGTTACGAGCCGGTCTCTGTTTA 1285
2259 TTGGCATGCAAGCTACGCGCTTAATTCGTACTGGGCTCAGTTTCGTAACC 2308
1286 TTGGCACGCAAGCTACGCGCTTAACTCGTACTGGGCACAGTTCCGTAGCC 1335
2309 AAAGCATTCAATGTTTCTTCAATGGATTGGCTCATGAGACAACAACCAAC 2358
1336 AAAACGTCCAATGTTACTTCAATGGATTAGCTCATGAGACCACGACTAAC 1385

FIG. 5a (8/8)

```
2359 CCTGGTGAGCCATTCTTTGTAGTTTCCAAATTTAGACCAAAATAACCTTT 2408
     ||||
1386 CCTG.....................................

2409 TCGTATAGTCACTAACAAAGATTTTTTACAGGAAATGATCGTTGCAAGTT 2458
                                 |||||||||| ||||||||
     ............................GAAATGATCGCTGCAAGTT 1408

2459 TCCGAGCGTTACTCTGTGAGGAGGACTTGAGGAAGAAGACACATGATTAA 2508
     |||||||||||||||||||||| ||      |||  |||   | |||  |
1409 TCCGAGCGTTACTCTGTGAGGAAGAACGCCTGAAAGAGATTTAAGATGAT 1458

2509 AGCTGGATTATTCGTATAACTCAATAATGTTCCTTATCTTTTTTTTTATT 2558
         |   |||  |   | | | |
1459 CAAAGCTGGATTATTCGTATTTACTC 1484

2559 ATACCTTTTTT 2569
```

FIG. 5b (1/3)

Alignment of the putative polypeptides encoded by the *A.thaliana* A6 gene and the *B.napus* cDNA A6.

Percent Similarity: 90 Percent Identity: 83

```
At    1 MSLLAFFLFTILVFSSSCCSATRFQ.GHRYMQRKTMLDLASKIGINYGRR 49
        |||||::||||.:|||. || .|||:|:||||:||||||||||||.
Bn    1 .....FFLFTLVVFSSTSCSAVGFQHPHRYIQKKTMLELASKIGINYGRQ 45

     50 GNNLPSPYQSINFIKSIKAGHVKLYDADPESLTLLSQTNLYVTITVPNHQ 99
        |||||||||||||| ||||||||||||||||||||||||.||.||
     46 GNNLPSPYQSINFIKLIKAGHVKLYDADPESLTLLSQTNLYVTIAVPTHQ 95
```

FIG. 5b (2/3)

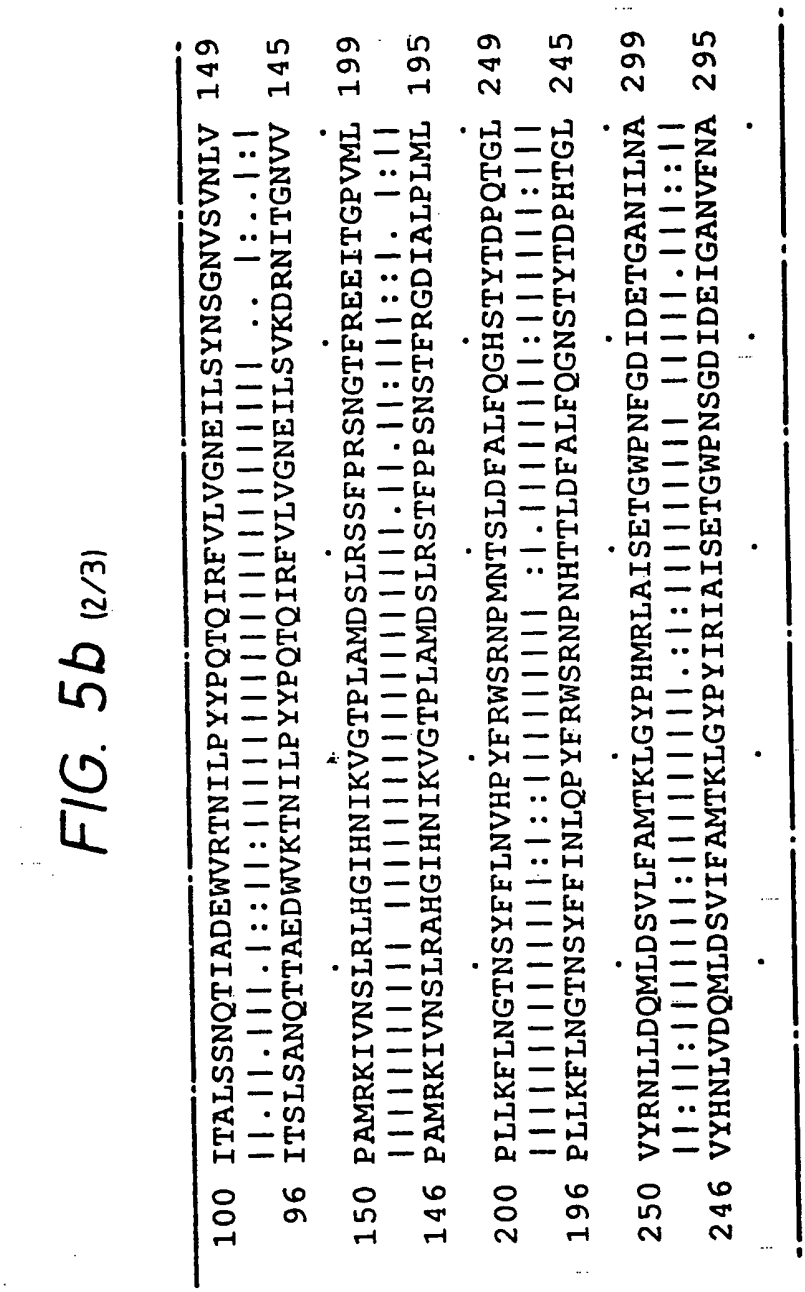

100 ITALSSNQTIADEWVRTNILPYYPQTQIRFVLVGNEILSYNSGNVSVNLV 149
96 ITSLSANQTTAEDWVKTNILPYYPQTQIRFVLVGNEILSVKDRNITGNVV 145
150 PAMRKIVNSLRLHGIHNIKVGTPLAMDSLRSSFPRSNGTFREEITGPVML 199
146 PAMRKIVNSLRAHGIHNIKVGTPLAMDSLRSTFPPSNSTFRGDIALPLML 195
200 PLLKFLNGTNSYFFLNVHPYFRWSRNPMNTSLDFALFQGHSTYTDPQTGL 249
196 PLLKFLNGTNSYFFINLQPYFRWSRNPNHTTLDFALFQGNSTYTDPHTGL 245
250 VYRNLLDQMLDSVLFAMTKLGYPHMRLAISETGWPNFGDIDETGANILNA 299
246 VYHNLVDQMLDSVIFAMTKLGYPYIRIAISETGWPNSGDIDEIGANVFNA 295

FIG. 5 b (3/3)

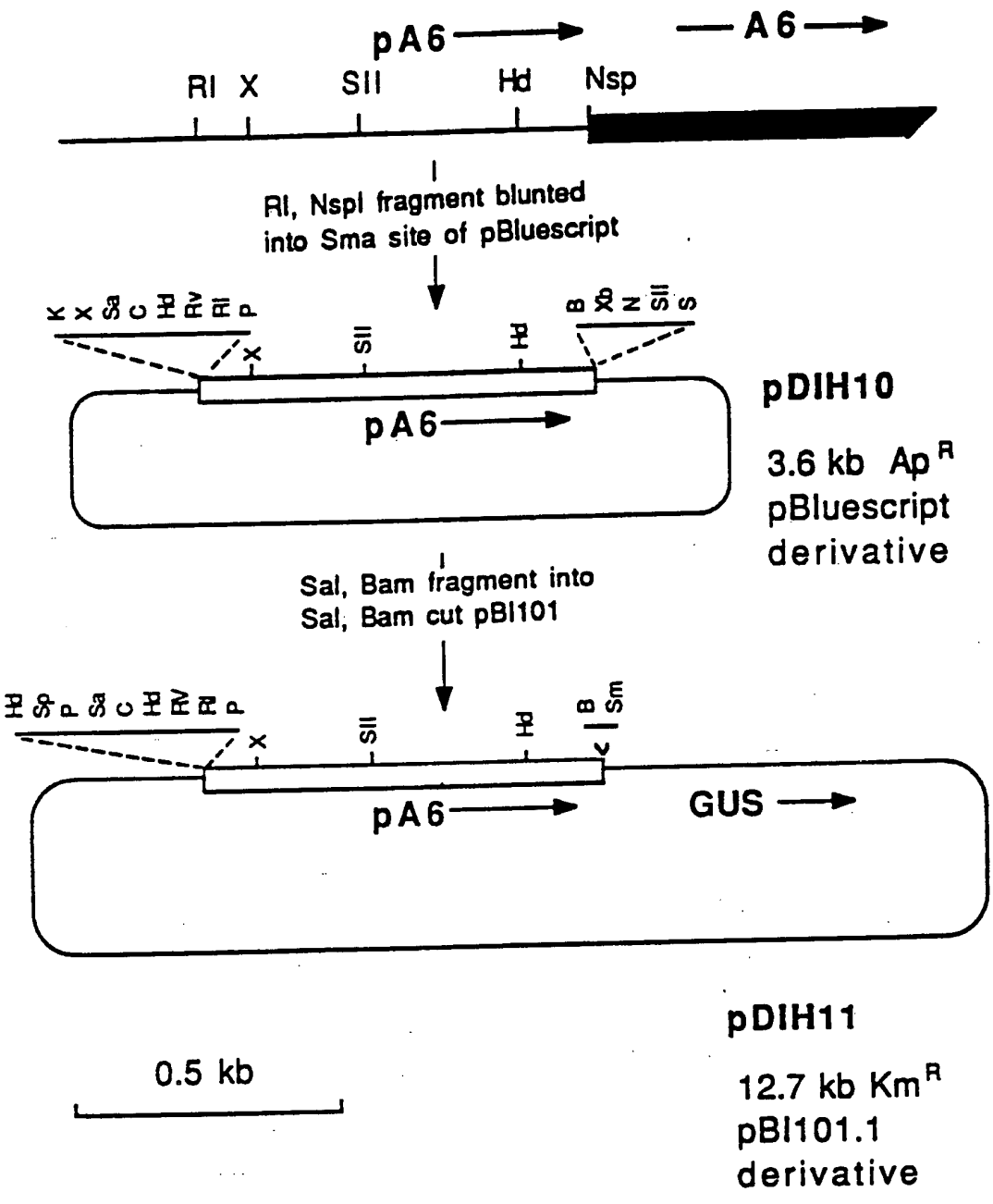
```
300 ATYNRNLIKKMSASPPIGTPSRPGLPIPTFVFSLFNENQKSGSGTQRHWG 349
    ||||||||||||.|.|||||||.|||  |||||||||||||||.|.|||||||||
296 ATYNRNLIKKMTATPPIGTPARPGSPIPTFVFSLFNENKKPGSGTQRHWG 345

350 IFDPDGSPIYDVDFTGQTPLTGFNPLPKPTNNVPYYKGQVWCVPVEGANET 399
    |:.|||.||||:||||||.|||||||||||||||||||||||||||||||||
346 ILHPDGTPIYDIDFTGQKPLTGFNPLPKPTNNVPYYKGQVWCVPVEGANET 395

400 ELEETLRMACAQSNTTCAALAPGRECYEPVSIYWHASYALNSYWAQFRNQ 449
    ||||.|||||||.|||||||||.|||||||||||:|||||||||||||.|
396 ELEEALRMACARSNTTCAALVPGRECYEPVSVYWHASYALNSYWAQFRSQ 445

450 SIQCFFNGLAHETTTNPGNDRCKFPSVTL 479
    .:||:|||||||||||||||||||||||
446 NVQCYFNGLAHETTTNPGNDRCKFPSVTL 474
```

33/39 FIG.6



Key to DNA sequences

A6 coding region

Cloned A6 promoter fragment

34/39

FIG. 7a

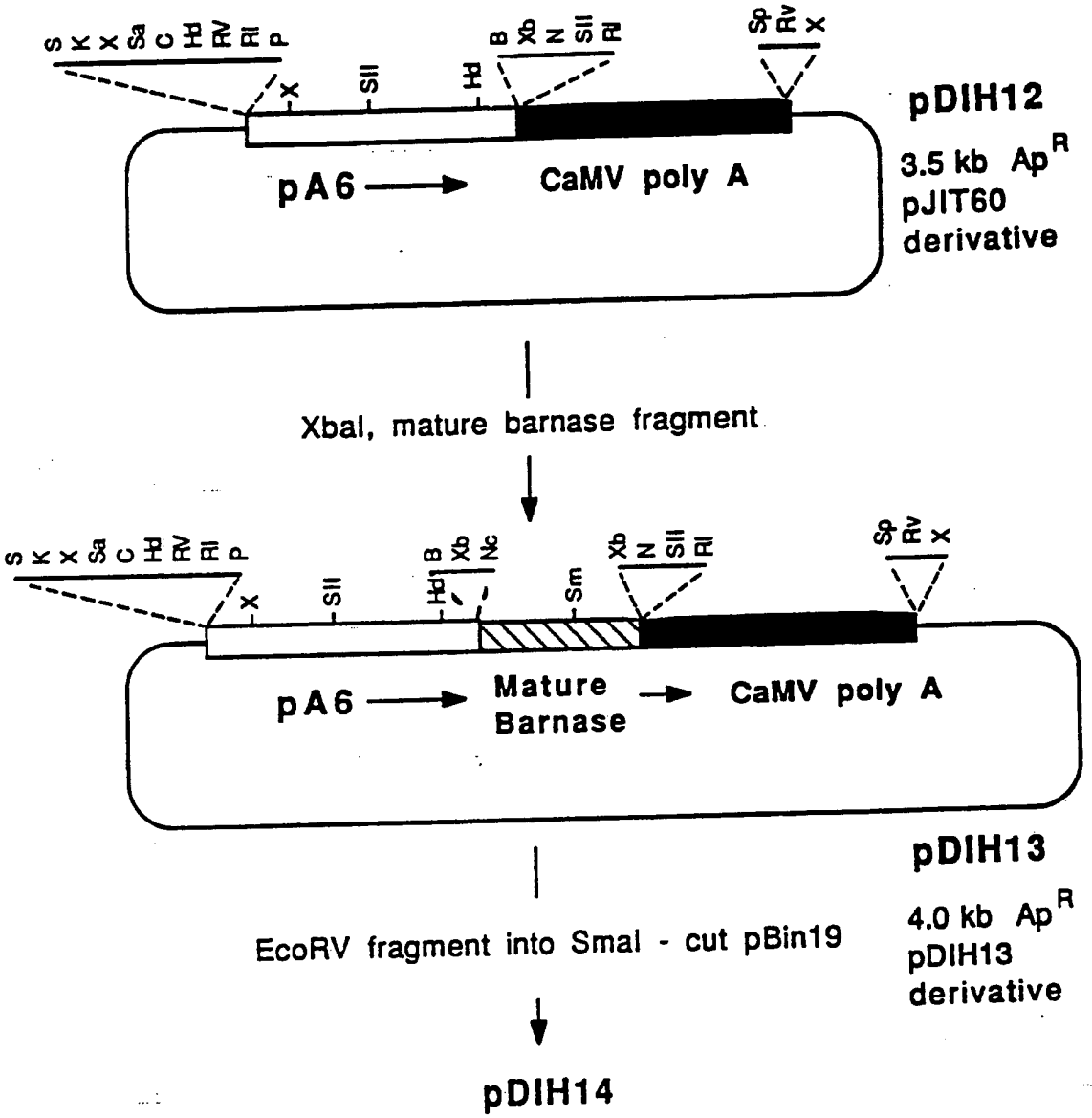

pA6
CaMV poly A
pDIH12
3.5 kb Ap^R
pJIT60
derivative
XbaI, mature barnase fragment
pA6
Mature
Barnase
CaMV poly A
pDIH13
4.0 kb Ap^R
pDIH13
derivative
EcoRV fragment into SmaI - cut pBin19
pDIH14
1 kb
Key to DNA
sequences
promoter
polyA signal

FIG. 7b

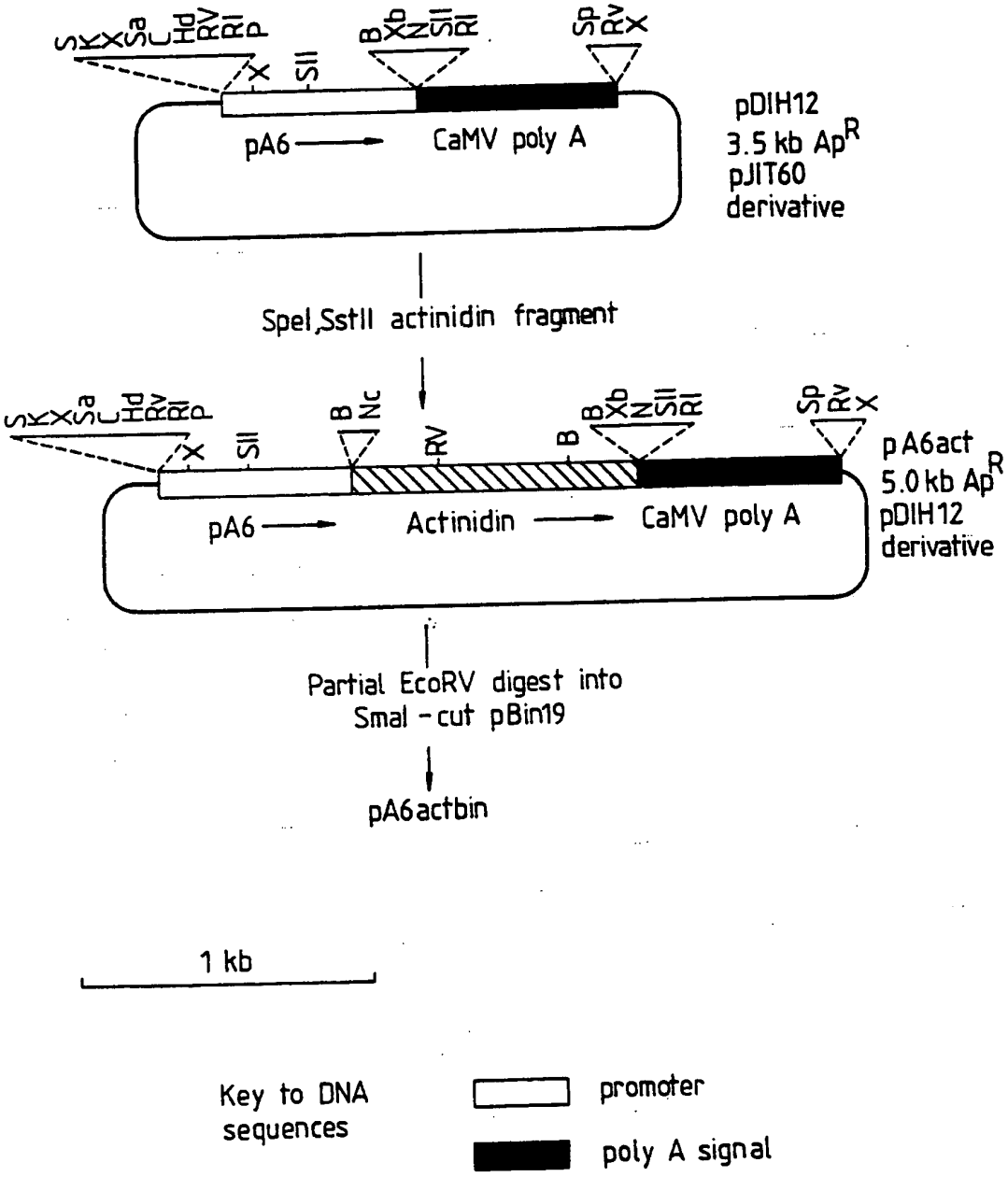

pA6
CaMV poly A
pDIH12
3.5 kb Ap^R
pJIT60
derivative
SpeI,SstII actinidin fragment
pA6
Actinidin
CaMV poly A
pA6act
5.0 kb Ap^R
pDIH12
derivative
Partial EcoRV digest into
SmaI - cut pBin19
pA6actbin
1 kb
Key to DNA sequences
promoter
poly A signal

FIG.8 (1/3)

a) Fusion of the A9 promoter to the A.thaliana callase gene

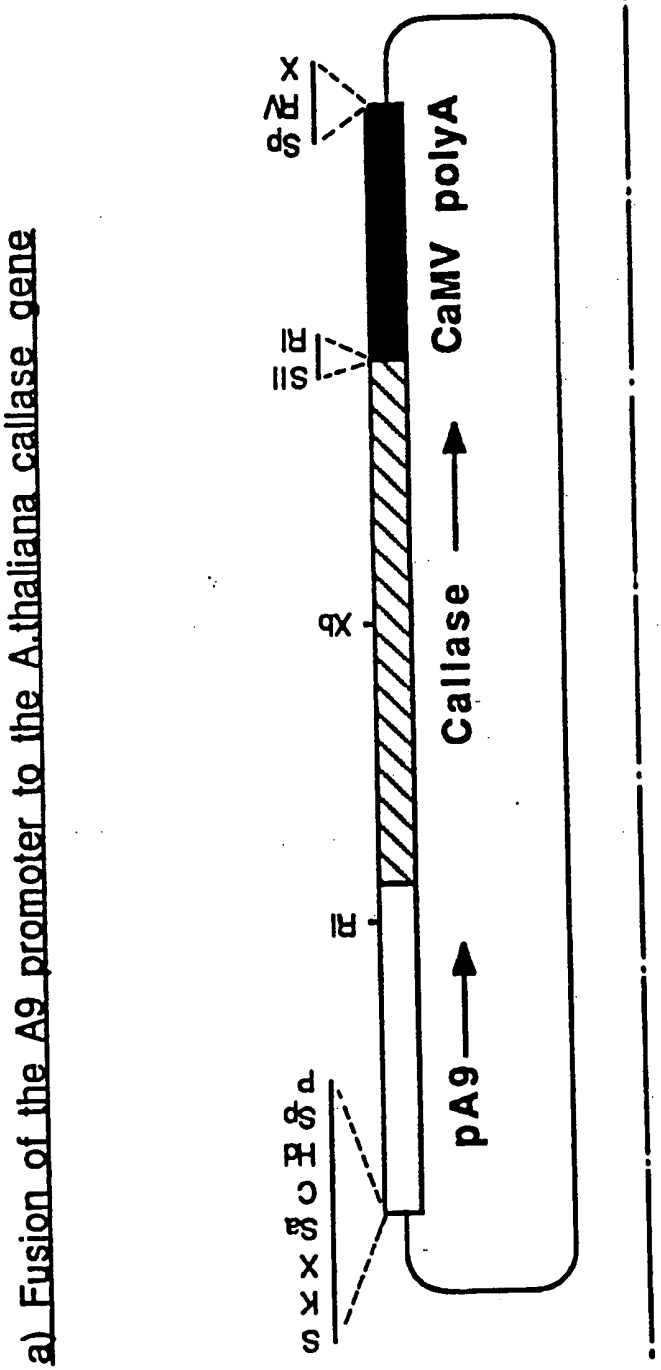


FIG.8 (2/3)

b) Fusion of the A6 and A9 promoters to C-terminal truncated callase

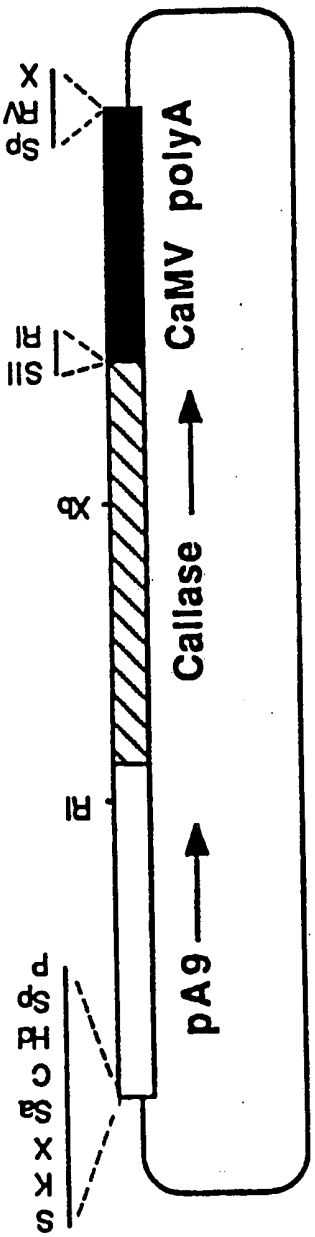


FIG. 8 (3/3)

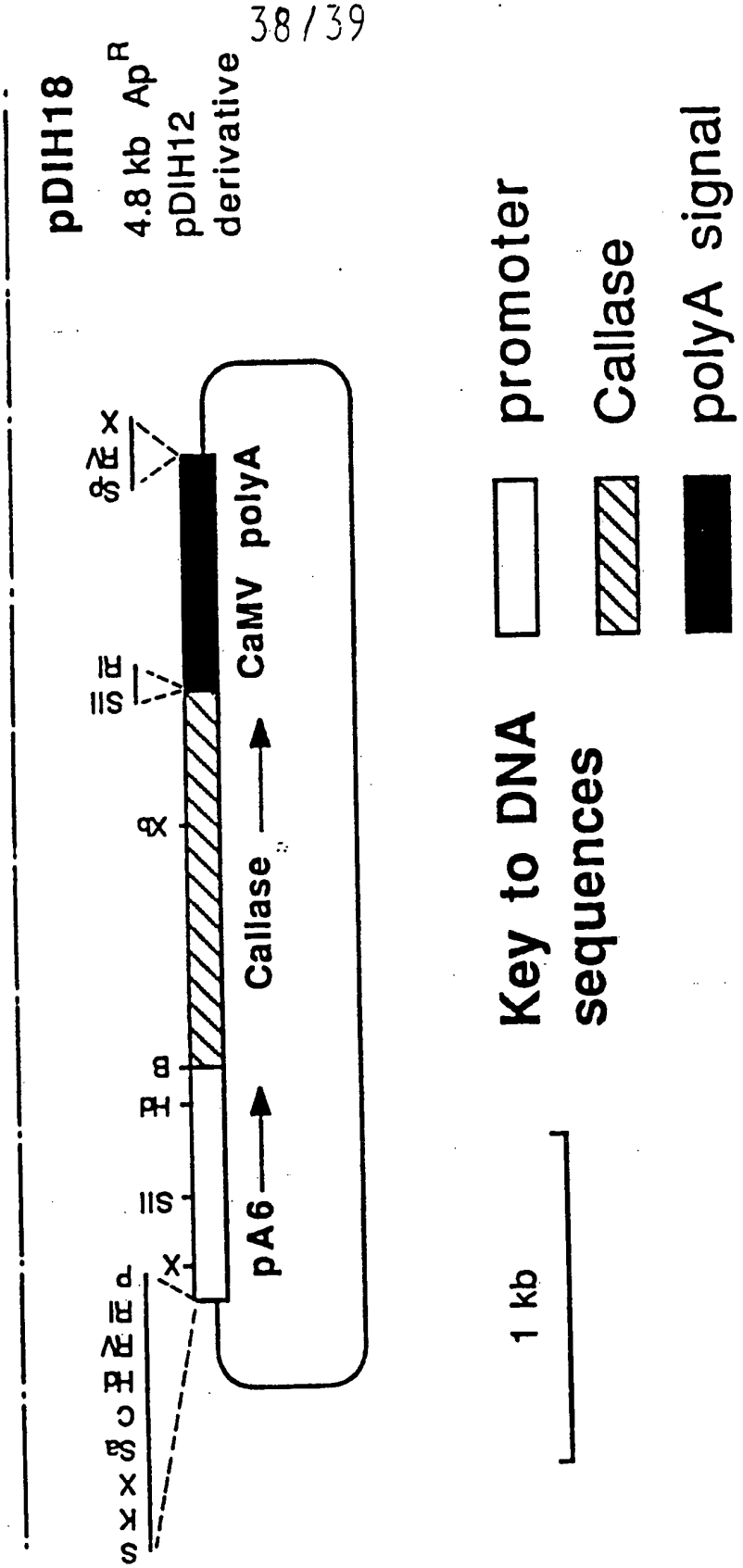
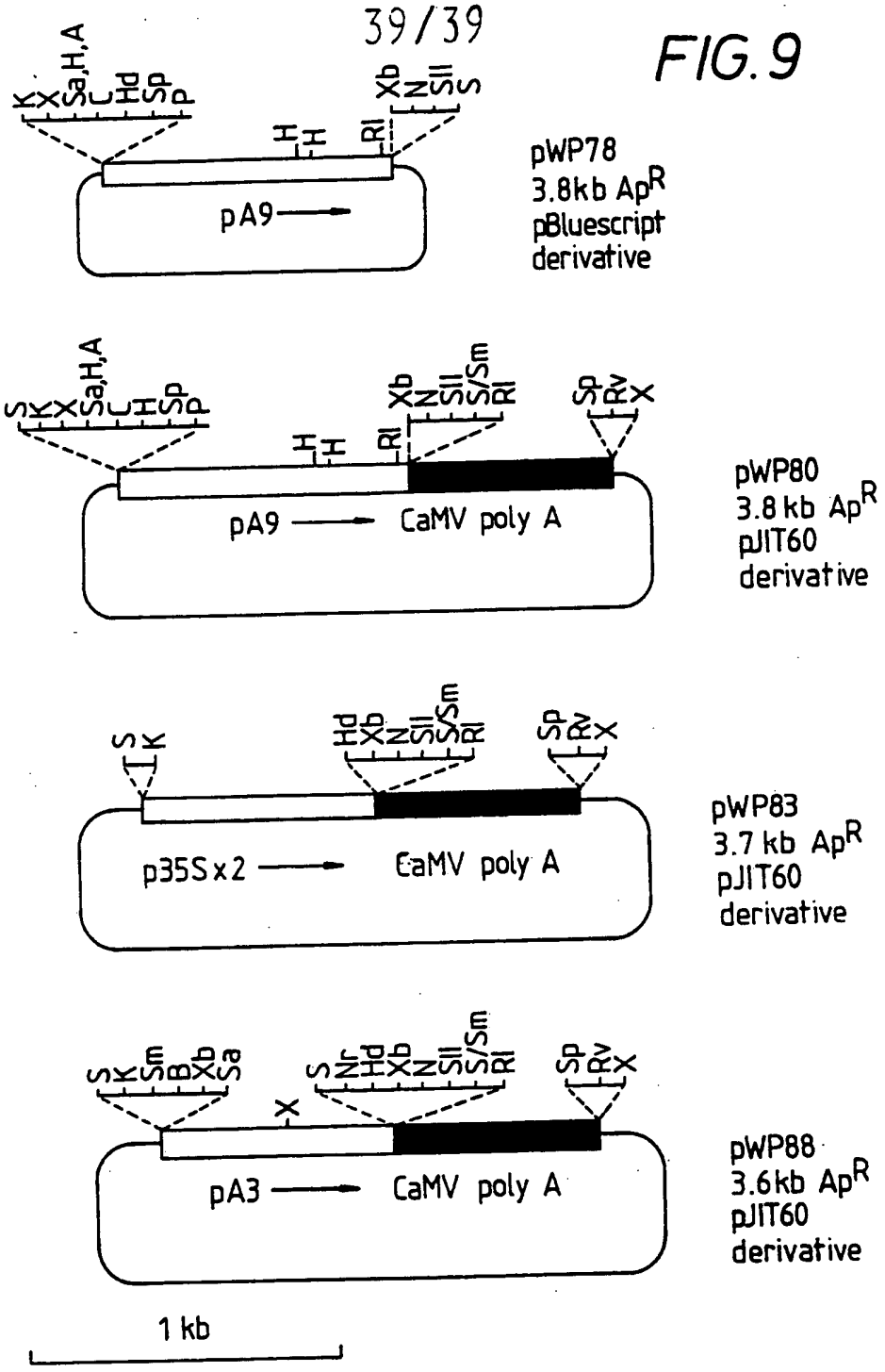

pDIH18
4.8 kb Ap^R
pDIH12
derivative
CaMV polyA
Callase
pA6
promoter
Callase
polyA signal
Key to DNA sequences
1 kb

FIG. 9


pA9
pWP78
3.8kb Ap^R
pBluescript
derivative
pA9
CaMV poly A
pWP80
3.8 kb Ap^R
pJIT60
derivative
p35S x2
CaMV poly A
pWP83
3.7 kb Ap^R
pJIT60
derivative
pA3
CaMV poly A
pWP88
3.6kb Ap^R
pJIT60
derivative
1 kb
Key to DNA sequences
promoter
poly A signal

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01354

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/56; C12N9/24; C12N15/82; C12N9/00
C12N15/11; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
O,X	J EXP. BOT., ANNUAL MEETING OF THE SOCIETY FOR EPERIMENTAL BIOLOGY, BIRMINGHAM, ALABAMA, USA, APRIL 7-12, 1991. vol. 42,, 1991, 238 SUPPL. page 40 PAUL, W., ET AL. 'Aspects of the molecular biology of anther development' see abstract P8.24 ---	1-3, 38-40
X	J. EXP. BOT.,1990 ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY vol. 41, 1990, SUPPL. page P5-3 SCOTT, R., ET AL. 'Identification of genes exhibiting cell-specific and temporal regulation in developing anthers of Brassica-napus' see abstract P5.09 --- -/--	1-3, 38-40

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30 OCTOBER 1992	16. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
O,X	ABSTRACTS VIITH INTERNATIONAL CONGRESS ON PLANT TISSUE AND CELL CULTURE.1990, JUNE 24-29, AMSTERDAM, NL. page 46 BARGHCHI, M., ET AL. 'Genetic engineering of Arabidopsis' see the abstract A2-10	1-47
X	EP,A,0 392 225 (CIBA-GEIGY) 17 October 1990 see example 32	1,5,24, 26,34, 37,38-44
X	EP,A,0 418 695 (CIBA-GEIGY) 27 March 1991 see page 38; example 9	1,45-47
P,X	PLANT MOLECULAR BIOLOGY. vol. 17, no. 2, 1991, DORDRECHT, THE NETHERLANDS. pages 195 - 207 SCOTT, R., ET AL. 'Patterns of gene expression in developing anthers of Brassica napus' see figure 5; table 2	1-3, 38-40
P,X	WO,A,9 211 379 (NICKERSON INTERNATIONAL SEED) 9 July 1992 cited in the application see example 5	1,5, 36-47
A	EP,A,0 344 029 (PLANT GENETIC SYSTEMS) 29 November 1989 see whloe document particularly page 4 line 18 - page 7 line 4	1-47
A	WO,A,9 008 828 (PALADIN HYBRIDS) 9 August 1990 see the whole document	1-47
A	PLANT PHYSIOLOGY.SUPPLEMENT, ANNUAL MEETING AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, JULY 29 - AUGUST 2, 1990 vol. 93, no. 1, May 1990, ROCKVILLE, MD, USA. page 131 DEL CAMPILLO, E., ET AL. 'Cell wall hydrolases in anther and abscission zones' see abstract 771	1

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
O,A	J. EXP. BOT., ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY, BIRMINGHAM, ALABAMA, USA, APRIL 7-12, 1991. vol. 238, 1991, SUPPL. page 46 HODGE, R.P., ET AL. 'A9 - a tapetum-specific gene' see abstract P8.55	1-47

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201354 SA 62495

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/10/92 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0392225	17-10-90	AU-A- 5218390	27-09-90
		CA-A- 2012778	24-09-90
		JP-A- 3035783	15-02-91
EP-A-0418695	27-03-91	AU-A- 6245790	21-03-91
		CA-A- 2025083	14-03-91
		JP-A- 3112488	14-05-91
WO-A-9211379	09-07-92	AU-A- 9133791	22-07-92
EP-A-0344029	29-11-89	AU-B- 621113	05-03-92
		AU-A- 3537189	24-11-89
		WO-A- 8910396	02-11-89
		JP-T- 2503988	22-11-90
WO-A-9008828	09-08-90	AU-A- 5037290	24-08-90
		EP-A- 0456706	21-11-91
		JP-T- 4504355	06-08-92